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SURVEY ON CARCINOEMBRYONIC ANTIGEN (CEA) DETECTION IN NORMAL AND NEOPLASTIC HUMAN TISSUE BY USING PEROXIDASE-ANTI-PEROXIDASE (PAP) TECHNIQUE

by

(C) LAITH K. DABBAGH

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

IN

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled SURVEY ON CARCINOEMBRYONIC ANTIGEN (CEA) DETECTION IN NORMAL AND NEOPLASTIC HUMAN TISSUE BY USING PEROXIDASE-ANTI-PEROXIDASE (PAP) TECHNIQUE submitted by LAITH K. DABBAGH in partial fulfillment of the requirements for the degree of Master of Science in Medical Laboratory Science in the Department of Pathology.

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Dedicated to my esteemed parents,

Mary and Karim Dabbagh, who have

provided me with their love and moral support

enabling me to carry out this study.

ABSTRACT

Carcinoembryonic Antigen (CEA), a glycoprotein with a molecular weight of 180,000 to 200,000 daltons was first described by Gold and Freedman. It was then believed that CEA was present only in cancers of gastrointestinal origin and fetal digestive organs in the first two trimesters of gestational age, and, for this reason, it was called carcinoembryonic antigen. It has since been demonstrated that CEA is present not only in sera of patients with enteric carcinomas but also in those patients with extragastrointestinal carcinomas, heavy smokers, in some normal people, and also changes with sex and age, in males higher than in females and in older individuals higher than in younger individuals.

Peroxidase antiperoxidase (PAP), a highly sensitive technique, has been used in this study to detect CEA at the cellular level. CEA was detected not only in malignant neoplasms of gastrointestinal tumors, but also in benign gastrointestinal tumors, and in extragastrointestinal neoplasms arising from the breast, lung, kidney, transitional epithelium, ovary, cervix, prostate, testis, thyroid, parathyroid, adrenal gland, liver, salivary gland and pancreas.

CEA was also detected in inflammatory conditions of the bowel such as ulcerative colitis and Crohn's disease, in colonic and gastric polyp and in normal breast, gastrointestinal epithelium, kidney tubule, liver, and prostate.

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
	A. Historic Review	1
	B. Physiochemical properties of CEA	2
	C. Cross Reacting Antigens	4
	1. Nonspecific Cross-Reacting Antigen (NCA)	4
	2. Colon Carcinoma Antigen III (CCA III)	5
	3. Nonspecific Cross Reacting Antigen II (NCA II)	6
	D. Metabolism of CEA	6
	E. Current Status of CEA	8
II.	MATERIALS AND METHODS	10
	A. Materials	10
	B. Immunoperoxidase Techniques	11
	1. Enzyme Conjugated Method	12
	2. Non-Conjugated Method	14
	C. Advantges and Disadvantages of Peroxidase-Anti-Peroxidase (PAP)	17
	D. Substrate Chromogens	17
	E. Fixative	18
	F. Specificity and Sensitivity of PAP Technique	19
	1. Specificity	19
	a) Method Specificity	19
	b) Antibody Specificity	20
	2. Sensitivity	21
	G. Application of PAP Technique	22

CHAPTER PAGE

III. OBSERVATIONS AND RESULTS 24

A. Gastrointestinal Tract and Associated Glands 24

B. Thyroid, Parathyroid and Adrenal Glands. 29

C. Urinary System 31

D. Lung 33

E. Breast 35

F. Female Reproductive System 35

G. Male Reproductive System 37

IV. DISCUSSION AND CONCLUSION. 40

A. Gastrointestinal Tract and Associated Glands 40

B. Thyroid, Parathyroid and Adrenal Glands. 44

C. Urinary System 46

D. Lung 47

E. Breast 47

F. Female Reproductive System 48

G. Male Reproductive System 49

FIGURES. 51

BIBLIOGRAPHY 67

LIST OF TABLES

TABLE		PAGE
I.	Immunohistochemical Staining of CEA in Normal and Neoplastic Gastrointestinal Tract and Associated Glands	25
II.	Immunohistochemical Staining of CEA in Normal and Neoplastic Thyroid, Parathyroid and Adrenal Tissue	30
III.	Immunohistochemical Staining of CEA in Normal and Neoplastic Urinary System Tissue	32
IV.	Immunohistochemical Staining of CEA in Normal and Neoplastic Lung Tissue	34
V.	Immunohistochemical Staining of CEA in Normal and Neoplastic Breast Tissue	34
VI.	Immunohistochemical Staining of CEA in Normal and Neoplastic Female Reproductive System.	36
VII.	Immunohistochemical Staining of CEA in Normal and Neoplastic Male Reproductive System.	38

LIST OF FIGURES

FIGURE		PAGE
1	Direct Immunoperoxidase Method	51
2	Indirect Immunoperoxidase Method	52
3	Hybrid Antibody Method	53
4	Enzyme Bridge Method	54
5	Peroxidase-Antiperoxidase Technique	55
6	Nonspecific Background with PAP and Peroxidase-Labeled Antibodies	56
7	Immunoperoxidase Staining of a Normal Small Bowel	57
8	Immunoperoxidase Staining of a Microfollicular Adenoma of the Thyroid	57
9	Immunoperoxidase Staining of an Ulcerative Colitis of the Colon	58
10	Immunoperoxidase Staining of a Moderately Well Differentiated Adenocarcinoma, Dukes Class B, of the Colon	58
11	Immunoperoxidase Staining of a Tubular Adenoma in a Colonic Polyp	59
12	Immunoperoxidase Staining of a Benign Pleomorphic Adenoma of the Salivary Gland	59
13	Immunoperoxidase Staining of a Normal Pancreas	60
14	Immunoperoxidase Staining of an Active Liver Cirrhosis	60
15	Immunoperoxidase Staining of a Liver Cell Adenoma	61
16	Immunoperoxidase Staining of a Metastatic Mixed Papillary and Follicular Carcinoma of the Thyroid	61
17	Immunoperoxidase Staining of a Pheochromocytoma of the Adrenal Gland	62
18	Immunoperoxidase Staining of a Renal Cell Carcinoma of the Kidney	62

FIGURE PAGE

19 Immunoperoxidase Staining of a Transitional Cell Carcinoma, Grade IV, of the Urinary Bladder. 63

20 Immunoperoxidase Staining of a Small Cell Anaplastic Carcinoma of the Lung 63

21 Immunoperoxidase Staining of an Infiltrating Duct Carcinoma of the Breast. 64

22 Immunoperoxidase Staining of a CIN III of the Cervix 64

23 Immunoperoxidase Staining of a CIN III of the Cervix 65

24 Immunoperoxidase Staining of a Normal Testis 65

25 Immunoperoxidase Staining of a Terotocarcinoma of the Testis 66

LIST OF ABBREVIATIONS

CEA	Carcinoembryonic Antigen
PAP	Peroxidase-Antiperoxidase
CCA III	Colon Carcinoma Antigen III
NCA	Nonspecific Cross Reacting Antigen
NCA II	Nonspecific Cross Reacting Antigen II
NGP	Normal Glycoprotein
DAB	Diaminobenzidine
PCA	Perchloric Acid
FNPS	4,4,-Difluro,3,3,Dinitrodiphenyl Sulfone
Ig	Immunoglobulin
HRP	Horse Radish Peroxidase

I. INTRODUCTION

A. Historic Review

The Carcinoembryonic Antigen (CEA) of the human digestive system was described by Gold and Freedman (Gold and Freedman, 1964, 1965) as a "protein-polysaccharide complex present exclusively in adenocarcinomas derived from the digestive tract epithelium and in the digestive organs of foetuses aged two to six months."

CEA was so named because of its presence in neoplastic and embryonic gastrointestinal tissues.

It was first believed that CEA was present only in cancers of gastrointestinal (GI) origin and fetal digestive organs in the first two trimesters of gestation, but it has since been demonstrated that CEA is present not only in sera of patients with enteric carcinomas but also in those of patients with extragastrointestinal carcinomas, such as lung, breast, urinary bladder, prostate and bone (Denk et al., 1972, Rule and Kirch, 1976, Concannon et al., 1973) and in many normal and neopastic adult tissue extracts such as normal pooled sera, liver, pancreas, kidney, uterus, ovary, suprarenal gland and lung. The highest level of CEA activity is, however, in carcinomas originating from gut endoderm, i.e. carcinoma of the gastrointestinal tract, lung, and liver (Khoo et al., 1973).

Heavy cigarette smoking (Merill et al., 1981), inflammatory gastrointestinal disease, gastrointestinal ulcers, GI polyps, hepatic cirrhosis (Concannon et al., 1973), breast fluid discharge in benign and malignant breast diseases and in milk of lactating women (Kahana

et al., 1981), amniotic fluids at different stages of pregnancy (Gadler et al., 1978), and cerebrospinal fluid (Dearnaly et al., 1980) have been associated with elevated CEA levels.

Plasmal CEA levels are higher in males than females and are higher in older than younger individuals (Beavdonnel et al., 1981).

B. Physiochemical Properties of CEA

CEA is an acidic glycoprotein molecule, soluble in water, perchloric acid (PCA) and 50% saturated ammonium sulfate, but insoluble in ethanol. It has a molecular weight of 180,000 to 200,000 daltons, with electrophoretic mobility of a serum B-globulin under mildly alkaline conditions (pH 8.6), and a sedimentation coefficient of 6.9 to 8 S (Fritsche and Mach, 1976, Fuks et al., 1974, Pustaszeri et al., 1972, Gold et al., 1978, Egan et al., 1977). It is most stable at -20°C and 4°C, and at 37°C has a degradation rate of 1.4%/day. CEA is sensitive to enzymatic digestion with trypsin (approximately 55% loss) and extraction with perchloric acid PCA (more than 70% loss). The major amino acid residues of CEA include aspartic acid or asparagine, glutenic acid or glutamine, threonine and serine. Aspartic acid (including asparagine) is the most abundant amino acid, and lysine the single NH_2-terminal amino acid. Other amino acids found in variable concentrations are proline, glycine, alanine, valine, isoleucine, Leucine, tyrosine, phenylalanine, lysine, histidine and arginine (Newman et al., 1974). The NH_2-terminal sequence of the first 25 amino acids of CEA from colon lavages of

normal people is Lys-Leu-Thr-Ile-Glu-Ser-Thr-Pro-Phe-(Asn)-Val-Glu-Gly-Lys-Glu-Val-(Leu,Ile)-(Leu,Ile)-(leu-Ile-val-(His,Arg?)-?-Leu,Ile) (Shiveley et al., 1977). These amino acids constitute approximately 30 to 46% of the CEA molecule. The remaining 45 to 57% is carbohydrate which is 30 to 36% N-acetylglucosamine, 21 to 27% galactose, 13 to 20% mannose and 13 to 20% fucose and traces of N-acetyl galactosamine (Egan et al., 1976, Pusztaszeri et al., 1972). Carbohydrate moieties are distributed throughout the molecule (Egan et al., 1976).

The CEA molecule appears to have a single-chain structure with multiple intra-chain disulfide bonds, and the conformational arrangement provided by the disulfide bridges is of some importance in determining the immunoreactivity of CEA. Antigenic activity increases with increasing mannose content (Deepika et al., 1981, Slayter and Coligan, 1976, Hammarstrom et al., 1975). The CEA molecule, at neutral pH, appears ultrastructurally as a twisted rod-shaped particle, about 9x40 nm. Conformation of the molecules is dependent on pH. At pH4, near the average isoelectric point, the molecule tends to be more globular. At pH9, it becomes more elongated, due to negative charges distributed throughout the molecule which repel each other (Slayter and Coligan, 1972, Gold et al., 1978). Thus CEA has a polypeptide backbone with a theoretical length of 220 nm which is folded in some way into a particle 40 nm long with the carbohydrate attached as multiple side chains, probably through asparagine linkages to glucosamine residues (Slayter and Coligan, 1972, Egan et al., 1977).

A number of physiochemical procedures have shown that even highly purified CEA preparations manifest a degree of both

intermolecular and intramolecular heterogeneity (Fuks et al., 1975, Hammarstrom et al., 1975, Elliot, 1978).

CEA derived from normal and from neoplastic colonic tissue has similar molecular weight, carbohydrate composition and protein composition. Both have lysine as the single amino terminus, both give a single peak in the cesium chloride density gradient with a density of 1.42, both are PAS positive and both give a line of identity with anti CEA in double diffusion (Shively et al., 1977, Fuitsche and Mach, 1976, Egan et al., 1977).

C. Cross Reacting Antigens

CEA has antigenic determinants that are shared with a family of closely related substances. Among the variety of glycoproteins bearing some antigenic relationship to CEA, nonspecific cross-reacting antigen (NCA), colonic carcinoma antigen III, (CCA-III) and nonspecific cross reacting antigen II (NCAII) are the most extensively characterized and are probably identical to other substances variously identified as CEA-associated protein (CEX), normal glycoprotein (NGP), and tumor-associated antigen (TEX) (Egan et al., 1977, Burtin et al., 1977).

1. Nonspecific Cross-Reacting Antigen (NCA)

NCA, a glycoprotein present in normal human tissue and human plasma, is neither organ nor tumor specific and thus is termed "nonspecific cross-reacting antigen." NCA can be detected in almost all organ extract, the highest concentration in fetal colon increasing with gestational age of the fetus (Pustaszer et al., 1972, Kleist et al., 1972).

High levels of NCA are found in normal and neoplastic liver, spleen, placental tissue, bone marrow, granulocytes, lactating women, amniotic fluids of normal and abnormal pregnancies and serum as well as the normal gut. In contrast to CEA, which is normally found in very low levels, in adults, and is a constituent of the cell membrane, the glycocalyx specifically (Gadler et al., 1980, Gadler et al., 1978, Kahana et al., 1981). NCA can cross react with CEA in radioimmunoassay (RIA) (Pustaszeri et al., 1972, Kleist et al., 1972).

Two characteristics differentiate NCA from CEA. NCA has a lower molecular weight, about 60,000 daltons, and a sedimentation constant between 3-4 S while CEA is 6.8 to 8.0 S. NCA and CEA have shared antigenic determinants which are either located on the protein or the polysaccharide moiety of the molecule.

Both NCA and CEA are resistant to lower pH, both are soluble in PCA, and periodic acid shift (PAS) positive, both have identical electrophoretic mobility, and both migrate toward the cathode. The protein content in CEA per dry weight is 30-46% while NCA is 66%. Carbohydrate composition is identical but concentration is variable (Pustaszeri et al., 1972, Kleist et al., 1972).

2. Colon Carcinoma Antigen III (CCA III)

CCA III is a B glycoprotein closely related to NCA. Its molecular weight is 60,000 daltons, and it has a similar amino acid composition to CEA though a different carbohydrate content (D-galactose and sialic acid are absent). CCA III expressed at least one antigenic determinant not present on CEA by radioimmunoassay (RIA) (Prinn et al., 1977). Normal circulating levels of CCA III are 1 to

2000 times higher than the comparable values of CEA. CCA III was found in colon carcinomas and initially thought to be a precursor of a subunit of CEA, but the presence of the amino acid methionine in CEA is not compatible with this hypothesis (Primus et al., 1977, Newman et al., 1974).

Interaction between CEA and CCA III will not occur at high ionic strength and it is maximal at low ionic strength (Primus et al., 1977, Newman et al., 1974).

3. Nonspecific Cross Reacting Antigen II (NCA II)

NCA II is a glycoprotein found in normal digestive mucosa. It appears to be a mucus-associated component, excreted in the lumen of the stomach and intestine. It is also found in fetal digestive mucosa and in digestive carcinoma. Meconium and feces of normal subjects contain elevated quantities of NCA II (Burton et al., 1977).

Immunofluorescent staining of normal colonic mucosa for the specific NCA II anti serum localized NCA II at the glandular luminal surface and within the goblet cells, suggesting that NCA II is a mucin-associated antigen (Primus et al., 1981, Leung et al., 1977).

NCA II can be extracted from normal colon by PCA digestion (Burtin et al., 1977). NCA II has a molecular weight of about 125,000 daltons (Primus et al., 1981).

D. Metabolism of CEA

The precise metabolism of the CEA molecule remains to be determined. Studies of CEA concentrations in the sera of patients who

that CEA is catabolized relatively rapidly, since serum concentrations postoperatively frequently fall to undetectable levels anywhere from 2 to 14 days after surgery (Minton et al., 1978, Gold et al., 1978).

The site of CEA catabolism in man remains unknown. Investigations in animals suggest that the liver is the major site for this process (Thomas, 1980). CEA appears in bile 47 minutes after injection intravenously (Thomas, 1980). About 50% of patients with severe benign hepatic disease, such as active alcoholic cirrhosis, chronic active and viral hepatitis, cryptogenic and biliary cirrhosis have elevated CEA levels. Patients with benign extrahepatic biliary obstruction may have increased plasma CEA. Benign and malignant liver tumors increase CEA plasma levels (Loewenstain and Zamcheck, 1978). Thus the liver appears to be essential in the metabolism and/or excretion of CEA.

There are at least three possible mechanisms by which protein, CEA may enter the bile. These are:

(1) by the detergent action of bile salts on the membrane of the canalicular face of the liver cell.

(2) by a specific receptor-mediated transport across the hepatocyte with discharge into the bile of the hepatocyte canalicular surface.

(3) by a nonspecific mechanism either involving pinocytosis or diffusion, in which the CEA passes through the fenestrated endothelium of the peribiliary capillaries and enters the periductular spaces where it is transported across the epithelial cell membrane either by pinocytosis or diffusion, and through the intercellular spaces (which

are not endowed with tight junctions) between the basal cytoplasmic projections (Thomas, 1980).

Recent studies have shown that desialylated glycoproteins may be extracted from the blood circulation by the liver in animals. The exposure of two galactose moieties is required for hepatic uptake of glycoprotein molecules. This is accomplished by removing termial sialic acid molecules, permitting binding by the hepatic plasma membrane binding site (Ashwell and Morell, 1974, Zamcheck et al., 1981). Native CEA (non desialylated) was found to be picked up first by Kupffer cells, desialated and then transferred to the hepatocytes (Thomas et al., 1977, Zamcheck et al., 1981, Thomas and Summer, 1977).

E. Current Status of CEA

It is now recognized that CEA can be detected in many human tumors and some normal human tissues. Since CEA is present in sera of some normal people as well as some cancer patients, a value of less than or equal to 2.5 ng/ml has been accepted as normal for the widely used radioimmunoassay method (Evans et al., 1979, Concannon et al., 1973, Zamcheck and Martin, 1981, Cauchi et al., 1981, Parente et al., 1981, Goslin et al., 1981, Waalkes et al., 1980, Dierksheide, 1981). Levels above this are found in association with various carcinomas, particularly those of gastrointestinal tract (Mach et al., 1978, Wayer et al., 1978, Sugarbaker et al., 1978), pancreas (Zamcheck et al., 1981), lung (Ford et al., 1981, Ahlemann et al., 1980, Krauss et al., 1981, Waalkes et al., 1980, Goslin et al., 1981), ovary and uterus

(Cauchi et al., 1981, Parente et al., 1981, Koch et al., 1980), breast (Frenchimont et al., 1977, Chu and Namoto, 1973, Staab et al., 1979, Moshakis et al., 1981), kidney, transitional epithelium, liver, salivary glands, and other benign tumors, in cigarette smokers and in 15% to 20% of subjects with inflammatory conditions such as ulcerative colitis, Crohn's disease, pancreatitis, liver and pulmonary infections (Neville, 1981, Zamcheck et al., 1981, Melia et al., 1981, Mavligit et al., 1978, Marton et al., 1978).

Any disease involving the liver or the use of hepatotoxic drugs may be associated with elevated CEA levels. Thus the possibilities of primary or secondary liver diseases or the use of hepatotoxic drugs must be eliminated before assuming that an elevated CEA level is due to a malignant or inflammatory gastrointestinal disease (Savrin et al., 1980).

At present CEA plasma levels are used as a long-term follow-up of colorectal carcinoma and other tumors in other sites (Mach et al., 1978, Mirton et al., 1978, Koch et al., 1981, Parente et al., 1981, Ahlemann et al., 1980, Chu and Namato, 1973). An increase in CEA plasma levels after resection of the tumor may indicate metastatic disease, that the tumor has not been completely resected, or that the tumor has reoccurred. Nonspecific causes that can raise CEA levels must be excluded.

Currently CEA can be detected in the tissue at the microscopic level by using formalin fixed, paraffin embedded tissue and using peroxidase-antiperoxidase technique.

II. MATERIALS AND METHODS

A. Materials

Sections from two hundred and ninety-four tissue blocks were stained by peroxidase-antiperoxidase technique (PAP) for CEA. The tissues examined included both normal and neoplastic tissue from various organs (Table I-VII, Chapter 3).

The tissue obtained by surgical biopsy was fixed in 10% buffered formalin and processed, using standard techniques, through alcohol and xylene to paraffin blocks. Thin sections (4-5 microns) cut from the blocks were picked up on plain glass slides, incubated overnight at fifty-five degrees and stained.

Antisera used for PAP staining and other reagents were obtained from commercial sources, i.e. (1) rabbit anti-human CEA from Cedarlane (Hornby, Ontario), (2) goat anti-rabbit IgG heavy and light chains from Coppal Laboratories (Cochranville, Pa.), (3) rabbit PAP from Cedarlane, (4) 3,4, 3, 4, tetroaminobiphenyl hydrochloride (diaminobenzidine, DAB) from Sigma (St. Louis, Mo.), (5) normal goat serum from Polysci (Markham, Ontario), (6) Hemotoxyline from Sigma, (7) Hydroxymethyl aminomethane (tris buffer from Sigma), (8) HCl from Sigma, (9) Sodium Chloride (NaCl from Sigma).

The buffer used for washing slides and diluting antisera was 0.2 M Tris Saline Buffer, which contained 0.2 M Tris (Hydroxymethyl Aminomethane), 0.1 normal HCl and 0.85% NaCl. A second buffer, 0.05 M Tris, was used for DAB reconstitution in the final stage of the PAP staining technique.

Harris Hemotoxylin which stained nuclei blue was used as a counter stain.

A moist chamber was used to incubate the slides with the antisera during reaction time.

Diatex was used for coverslipping. The coverslipped, PAP stained hematoxylin counter-stained slides were examined by conventional light microscopy.

B. Immunoperoxidase Techniques

Tissue pathologic diagnosis hinges on the microscopic morphology of thin tissue sections stained with hematoxylin and eosin. Special staining procedures can be of great value in tissue diagnosis by providing morphologic information regarding the functional status and perhaps histiogenesis of the examined tissue. Electron microscopy, Immunofluorescence and Histochemistry have also been of great help in tissue diagnosis. These later techniques, however, require specialized and expensive instruments and highly trained technical staff.

The immunofluorescence technique has been used widely in many different areas of Science (Sternberg, 1979). It has, however, many disadvantages which are:

(1) specialized microscopy is required (i.e. fluorescent microscope).

(2) stained slides cannot be conveniently stored and they are not readily available for repeated examination.

(3) Cryostat sections, with all the disadvantages of frozen section preparations, must be used

(4) The procedure is limited to fresh tissue, thus limiting its usefulness in diagnostic pathology.

(5) Nonspecific tissue autofluorescence may interfere with the interpretation of stained sections (Sternberger, 1979, Bocker, 1974, Taylor et al., 1981, Taylor, 1978).

Immunoperoxidase techniques, which are more sensitive and more specific than immunofluorescence, can be used on fresh and formalin-fixed tissues, do not require fluorescence microscopy and stained slides can be stored for repeated examination. Immunoperoxidase techniques can be adapted for many particular antigens.

Immunoperoxidase techniques can be divided into two main methods:

(1) Enzyme conjugated method

(2) Non-conjugated method

1. Enzyme Conjugated Method

An enzyme, peroxidase, is conjugated directly to an antibody by using 4, 4-Difluro-3,3 dinitrodiphenyl sulfone (FNPS) or glutaraldehyde (Sternberger, 1979). These agents bind the immunoglobulin (Ig, or antibodies) through N-terminal amino group or E amino group of lysine with the amino group of the peroxidase. Immunoglobulin usually has a number of immuno groups, for this reason FNPS reacts 66 times faster with immunoglobulins, particularly IgG, than with peroxidase. Thus it will form polymers of immunoglobulins which could be conjugated to one or more of peroxidase enzymes (Sternberger, 1979).

Gluteraldehyde is more reactive than FNPS because it is water soluble. Therefore there will be less unreacted Ig with peroxidase

enzymes, but aggregation of immunoglobulins is extensive. Immunoglobulins will form a polymer of a dimer or a trimer which is bound to one peroxidase enzyme or more. But in general the polymers with glutaraldehyde is less than in FNPS.

Both reagents form polymers of immunoglobulins which are conjugated to one or more or no peroxidase molecules. The immunoglobulin polymer and immunoglobulin polymer which is conjugated to peroxidase molecules cannot be separated from one another by gel filtration which can lead to false negatives (Sternberger, 1979).

Horseradish peroxidase (HRP) is heme enzyme isolated from horseradish roots. Carboxylate anions, amino groups, and imidazole groups have been postulated as possible heme-linked ligands in (HRP) (Tsoo et al., 1973).

HRP enzyme has been used widely because:

(1) the enzyme is easily detectable by a cytochemical method which can be applied to light microscope or electron microscope

(2) the enzyme is available in reasonably pure form

(3) conjugation with Ig will not affect its activity

(4) the enzyme is stable in neutral solutions.

A major disadvantage with the use of HRP is the presence of endogenous tissue peroxidase which can give high background or false positive staining when DAB is added. This endogenous tissue peroxidase can be blocked by pretreatment of the tissue with hydrogen peroxidase in methanol (Sternberger, 1979, Taylor, 1978).

The enzyme conjugated method can be direct, where HRP conjugated antibody binds directly to specific antigens (Ag) (Figure 1), or indirect where the primary unlabelled antibody binds to specific antigens and a secondary antibody enzyme conjugated to HRP binds to the primary antibody (Figure 2). A color reaction is carried out later.

2. Non-Conjugated Method

There are three different methods:

(1) enzyme hybrid antibody method

(2) enzyme bridge method

(3) peroxidase-antiperoxidase method (PAP).

Enzyme Hybrid Antibody Method. In the hybrid antibody method antibodies raised against the antigen (CEA) are cleaved enzymatically and reconstituted with similarly split antiserum derived from same species, but directed against horseradish peroxidase (Figure 3).

These two split antibodies are reconstituted and purified by solid-phase absorption to select for divalent molecules. The new antibody will have one valency directed against the antigen (CEA) and the other against peroxidase enzyme. In the final stage it requires the addition of free HRP then DAB. One disadvantage of this method is that the hybrid antibody preparations usually contains (in general) nonhybridized antibody, which reduces the sensitivity of the method.

Enzyme Bridge Method. This method involves a three-layer antibody technique with the final stage involving the addition of free HRP as shown in Figure 4.

First stage involves the addition of rabbit anti-human CEA, second stage involves the addition of excess goat anti-rabbit IgG,

third stage involves the addition of rabbit anti-horseradish peroxidase and then the addition of free horseradish peroxidase as a final stage. Then the DAB addition for the color reaction.

Peroxidase-Antiperoxidase Method (PAP). This very sensitive and specific method is approximately 100-1000 times more sensitive than immunofluorescence and also more sensitive than conjugated peroxidase method. (This will be discussed in more detail in Section F.) (Sternberger, 1979, Taylor et al., 1978, Burns, 1975, Bocker, 1974, Petrali et al., 1973).

The PAP method can be applied to formalin fixed and paraffin embedded tissue blocks. PAP procedure:

(1) Cut thin sections between 4-6 M and incubate overnight at 55°C.

(2) Bring sections down to water through:

(a) xylene two changes, five minutes in each

(b) graded ethyl alcohol

(c) water

(3) Block endogenous peroxidase by incubating in freshly made 1% Hydrogen Peroxide in methanol for ten minutes

(4) Wash in Tris buffer 0.20 M at pH 7.6 for ten minutes. Two changes.

(5) Incubate with normal goat serum, 1/20 dilution in Tris buffer, for ten minutes.

(6) Drain normal goat serum, incubate overnight in a moist chamber at 4°C with the primary antibody (1/1500 rabbit anti-human CEA).

(7) Wash in Tris buffer for 15 minutes, three changes on a shaker.

(8) Add secondary antibody or link antibody (goat anti-rabbit IgG at 1/40 dilution) for thirty minutes at room temperature (R.T.).

(9) Wash in Tris buffer for fifteen minutes, three changes on a shaker.

(10) Add rabbit-PAP at 1/30 dilution for thirty minutes at room temperature.

(11) Wash in Tris buffer for 15 minutes, three changes on a shaker.

(12) Add diaminobenzidene (DAB) to develop color, solution which contains 70% DAB and 0.006% Hydrogen Peroxide, freshly made, for six minutes.

(13) Wash slides in running tap water for at least twenty minutes.

(14) Counter stain in hematoxylin for one minute.

(15) Dehydrate and mount the slides (Figure 5).

Diaminobenzidene (DAB) is a carcinogen (Sternberger, 1979) and should be used with caution. Gloves should be worn and slides should be stained in a fume hood. Washing the slides with running tap water for 20 minutes is sufficient to clear the slides of DAB.

Controls Used.

(1) Known positive control (e.g. adenocarcinoma of colon) was used.

(2) Known negative control (e.g. normal thyroid tissue).

(3) Substitution of primary Ab with normal rabbit serum at 1/1500 dilution for each test (to be certain that nonspecific staining has been eliminated).

(4) No link antibody (to be certain that PAP is specific and is not linking directly to tissue).

(5) No PAP (to be certain that endogenous peroxidase is abolished).

C. Advantages and Disadvantages of Peroxidase-Antiperoxidase (PAP)

1. Advantages

(a) light microscopy is used

(b) stained slides are permanent and are available for repeated examination

(c) good tissue morphology

(d) unlimited technique can be applied to any specific antigen

(e) can be applied to ultrastructural studies

(f) it can localize a single antigen site, very high sensitivity.

2. Disadvantages

(a) presence of endogenous peroxidase in certain tissue (can be blocked by hydrogen peroxide in methanol or 0.1 M periodic acid) (Heyderman et al., 1977, Pearse, 1980).

(b) the use of carcinogen such as DAB.

D. Substrate Chromogens

The chromogen employed must be specific for peroxidase, producing a stable colored product with a well-defined visible absorption. The spectrum must be clearly seen by light microscopy. It should be compatible with counter-staining procedures and should not

be bleached out by dehydration and mounting methods. It should not inhibit nor denature the peroxidate enzyme. It should be nontoxic and noncarcinogenic, widely available, and inexpensive. Diaminobenzidine (DAB) meets all these requirements except that it is carcinogenic.

During the color reaction the peroxidase enzyme will break hydrogen peroxide and form molecular oxygen. Molecular oxygen oxidizes the DAB, an electron donor and forms a brown insoluble complex. E naphol pyronin can also be used giving a blue color but it bleaches out by dehydration with alcohol.

E. Fixative

An ideal fixitive for immunologic studies includes the following properties:

(1) preservation of antigen integrity so that it does not diffuse into the surrounding tissue

(2) non-interference with Ab/Ag reaction

(3) good tissue preservation with good morphology

(4) readily available

Ten percent buffered formalin can be and is widely used.

Other fixatives, such as Zenkers (contains potassium dichromate, mercuric chloride, glacial acid and distilled water) can be used but glutaraldehyde cannot because extensive cross linkage between antigen sites removes them from the immune reaction (Taylor et al., 1978).

F. Specificity and Sensitivity of PAP Technique

1. Specificity

The ability of a method to measure or detect one compound in a tissue without interference by other components in the tissue is termed "specificity." False positive results can be obtained if the method is not specific because other compounds are detected.

There are two types of specificity in immunoperoxidase and immunofluorescence:

(a) method specificity

(b) antibody specificity

(a) Method Specificity

(1) Labelling of Ab with enzyme yields antibody aggregation or polymerization. Antibody polymers will bind to nonspecific antigens by hydrophobic bonds, electrostatic attraction or salt linkages. Conjugation with Fluorescein Isothiocynate (FITC) results in a negative charge on the conjugated antibodies and thus allows for the formation of more antibody aggregates, hence more nonspecific binding in immunofluorescence. Nonspecific binding of antibody aggregates will give false positive results and high background. This is not encountered with PAP technique, hence there is no labelling process, therefore less background, higher specificity and higher sensitivity (Taylor et al., 1978, Sternberger, 1979, Burns, 1975).

(2) Fc receptors are found on various tissues, not only immunoglobulins. Thus the link antibody may attach directly to the tissue instead of to the primary antibody. This type of nonspecificity is encountered in all the methods, but it can be avoided to a certain

degree by preincubation with normal goat serum which coats Fc tissue receptors. Preincubation with normal goat serum will decrease background and increase sensitivity of the method.

The PAP method is quite sensitive and high dilution of the antisera can be used. The use of very diluted antisera will decrease nonspecific background staining.

(3) Endogenous tissue peroxidase should be blocked by the use of hydrogen peroxide in methanol to eliminate nonspecific color. Analogous tissue autoflourescence is not easily abolished in immunofluorescence systems because autofluorescence emitted is at the same wavelength or close to FITC emission.

(4) The secondary antibody should be pure when labelled with enzyme in both immunofluorescence and labelled immunoperoxidase technique. If nonspecific antibodies are present, they will be labelled and will bind to the tissue directly giving a "positive" reaction with IF or IP. This false positive reaction is not encountered with PAP since there are no labelled antibodies, also if there is a nonspecific antibody it will not give false positive, since the PAP will not bind the nonspecific antibody because it is not directed against the PAP complex (Figure 6).

(5) PAP can bind nonspecifically to tissue directly, especially to collagen or reticulin fibers. This nonspecific binding can be avoided by preincubation with normal goat serum.

(b) Antibody Specificity

(1) The presence of nonspecific, in addition to specific, determinants in the purified immunizing antigen will result in production

of antibodies which can react with the tissue that has the same non-specific antigenic properties.

(2) Impurities in immunizing antigens. The more pure the antigen, the more specific the antibody produced and the more sensitive the method. Impurities will result in production of nonspecific antibodies, and hence a false positive result.

(3) Cross-reaction of antibodies due to similarities between antigens, (e.g. between CEA and NCA, CCA III and NCA II) can result in a false positive result.

2. Sensitivity

Sensitivity is the ability of a method to measure or detect a small amount of a compound. False negative results can be obtained if the method is not sensitive because minute amounts of a compound may not be detected. The PAP method is a very sensitive procedure. It is about 100 to 1000 times more sensitive than immunofluorescence and about 20 times more sensitive than labelled immunoperoxidase or radioimmunoassay (RIA) (Sternberger, 1979, Taylor et al., 1978, Boker, 1974, Morianty et al., 1973).

PAP has high sensitivity because of very low background staining (see "specificity") and because antibody is not structurally altered by enzyme or FITC labelling.

PAP complex has a molecular ratio of peroxidase to antiperoxidase of 3/2. This proportion of 3 peroxidase to 2 antiperoxidase molecules is independent of the amount of peroxidase used in the preparation, the PAP complex has a fairly homogeneous composition and a very stable cyclic shape. The shape can be determined by negative

staining and examination with an electron microscope (Sternberger, 1979, Taylor et al., 1978). This cyclic shape will result in:

(1) an increase in sensitivity compared to immunofluorescence and labelled immunoperoxidase, because in the PAP method there will be three enzymes producing colored products for each single antigen.

(2) easy detection of the PAP antigen complex by electron microscope

(3) possibility of detection of a single reactive antigenic site.

Partial trypsin digestion (trypsinization) of the tissue sections will expose more antigenic sites and make the procedure even more sensitive but it may also increase background staining.

G. Application of PAP Technique

The PAP method, because it allows for the detection of a tissue product in situ, provides a bridge between the morphological and functional aspects of a tissue or tissue lesion. The PAP method has been applied to detect many antigens in routine surgical pathology such as:

(1) identification of the cell origin of an antigen like endocrine neoplasms

(2) histogenesis of a lesion

(3) detection of micro-organisms, e.g. detection of staphlococcus aureus antigen

(4) detection of antibodies

(5) distribution of cells, e.g. in the gut

(6) loss of normal antigen in a neoplasm

(7) identification of oncofetal antigens in neoplasms

(8) localization of immune complex in renal glomerulonephritis (Mukai, K., and Rosai, J., 1980, Taylor et al., 1978, Sternberger, 1979, Denk et al., 1977, Marucci et al., 1975, Erlandsen et al., 1975, Sternberger et al., 1970, Sinclair et al., 1981, Maciver et al., 1979, Detellis et al., 1979).

III. OBSERVATIONS AND RESULTS

Immunohistochemical staining of normal and neoplastic tissue for CEA was graded from negative to strongly positive depending on the intensity of the brown color produced and the number of cells stained. A negative stain is completely blue (hematoxylinophilic) with no brown staining (Figure 7). A weakly positive stain shows scattered fine brown cytoplasmic granules (Figure 8). A moderately positive stain shows stronger brown color with a larger number of fine cytoplasmic granules (Figure 9). A strongly positive stain shows numerous fine brown cytoplasmic granules in many cells (Figure 10).

A. Gastrointestinal Tract and Associated Glands

The results of PAP, CEA immunohistochemical staining of eight-nine tissue blocks from specimens of normal and neoplastic gastrointestinal tissue is shown in Table 1.

1. Esophagus

Normal esophagus was negative. Two of the three squamous carcinomas of the esophagus showed weak cytoplasmic positivity within tumor cells.

2. Stomach and Small Intestine

Five out of five gastric adenocarcinomas were strongly positive (numerous fine cytoplasmic brown granules). Two out of three normal stomach and two out of four small intestine were weakly positive, with fine supranuclear and membrane staining.

TABLE I

Immunohistochemical Staining of CEA
in Normal and Neoplastic Gastrointestinal Tract and Associated Glands

Tissue	Histopathology	Positivity/No. of Cases
Brunner's Glands	Normal	0/1
Esophagus	Normal	0/2
	Moderately well differentiated Squamous Carcinoma	2/3
Stomach and small intestine	Normal stomach	2/3
	Normal small intestine	2/4
	Tubulovillous Adenoma	1/1
	Gastric Adenocarcinoma	5/5
Colorectal	Normal	8/12
	Crohn's Disease	5/5
	Ulcerative Colitis	4/4
	Villous Adenoma	2/2
	Tubulovillous Adenoma	3/3
	Tubular Adenoma	3/3
	Juvenile Polyp	1/1
	Squamous Carcinoma	1/1
	Adenocarcinoma	12/12
Salivary	Normal	3/5
	Pleomorphic Adenoma	1/2
	Squamous Carcinoma	1/1
	Anaplastic Carcinoma	1/1
Pancreas	Normal Exocrine	0/4
	Islets of Langerhans	4/4
	Chronic Pancreatitis	1/1
	Metastatic Anaplastic Small Cell Carcinoma	0/1

Tissue	Histopathology	Positivity/No. of Cases
Liver	Normal	3/5
	Mild Cholestasis	1/1
	Cirrhosis	2/2
	Hepatocellular Adenoma	1/1
	Liver Cell Carcinoma	1/1
Gallbladder	Normal	0/1
	Adenosquamous Carcinoma	1/1

Normal tissue adjacent to an adenocarcinoma showed weak to moderate cytoplasmic staining. Parietal cells showed weak cytoplasmic staining, Paneth cells, chief cells and Brunner's glands were negative.

A single tubulovillous adenoma of the stomach showed moderate cytoplasmic staining.

Connective tissue and muscle was negative even when adjacent to a positively staining adenocarcinoma.

3. Colorectal

The mucosa of eight out of twelve normal large bowel specimens was weakly positive for CEA. Positivity was maximal in the top layer cells (villi) and decreased toward the bottom cells (glands). Goblet cells were negative (Figures 7, 11). Brush border and membrane staining were present together with some intraluminal staining.

All adenocarcinoma tested were strongly positive (Figure 10), positivity did not depend on the size of the tumor, or the presence or absence of metastases to adjacent lymph nodes. Nuclear staining was negative at all times.

The staining of normal bowel mucosa adjacent to an adenocarcinoma varied from weakly to moderately positive (cytoplasmic and glycocalyx staining).

Inflammatory conditions such as ulcerative colitis and Crohn's disease showed moderate supranuclear cytoplasmic, brush border and glycocalyx staining (Figure 9).

The intensity of CEA staining increased with the degree of inflammatory tissue damage.

Benign large bowel polyps, neoplastic polyps (tubular, tubulo-villous and villous) showed moderate cytoplasmic and membrane staining (Figure 11). A non-neoplastic juvenile retention polyp showed only weak positivity.

One anal squamous carcinoma was weakly positive. Membrane staining was stronger than cytoplasmic staining intensity. Staining intensity increased with keratinization.

Connective tissue, muscle and nerve tissue were negative.

4. Salivary Glands

Weak cytoplasmic CEA staining was present in intercalated ducts in three out of five normal salivary glands, large ducts, and acini were essentially negative with only few acini showing weak intraluminal and apical positivity.

One anaplastic carcinoma showed moderate cytoplasmic and glycocalyx staining of the tumor cells. Pleomorphic adenoma showed weak cytoplasmic and membrane bound CEA staining (Figure 12).

Normal tissue adjacent to the tumor was negative. A single primary salivary gland poorly differentiated squamous carcinoma was weakly positive.

5. Pancreas

The exocrine portion of the normal pancreas was negative for CEA localization, except for intralobular ducts which showed weak cytoplasmic positivity (Figure 7). A few acinar cells showed very weak cytoplasmic positivity.

Island cells endocrine were weakly positive, with fine intra-cytoplasmic granules (Figure 13).

Metastatic anaplastic small cell carcinoma of the pancreas was negative. Chronic pancreatitis stained similarly to normal pancreas with apical cytoplasmic staining and weak positive intralobular ducts staining.

6. Liver

Normal hepatocytes showed weakly positive perinuclear cytoplasmic staining. Hepatic cirrhosis showed higher cytoplasmic positivity and some weak staining in the canaliculi (Figure 14).

Hepatic cholestasis is associated with increased cytoplasmic staining. Well differentiated hepatoma showed a slight increase in cytoplasmic CEA. A liver cell adenoma showed a marked increase in cytoplasmic CEA compared to normal liver, but the CEA positivity differed from one cell to another, and ranged from very weak to moderate staining of CEA (Figure 15).

Well differentiated adenosquamous carcinoma primary in the gallbladder showed weak cytoplasmic and membrane staining for CEA. Positivity increased with the degree of tumor cell keratinization.

B. Thyroid, Parathyroid and Adrenal Glands

The result of immunohistochemical staining for CEA is shown in Table II.

1. Thyroid Gland

Eight normal thyroid gland sections, three Hashimotos, six Nodular Goiter and one Graves disease were examined and all were negative (Figure 8).

TABLE II

Immunohistochemical Staining of CEA
in Normal and Neoplastic Thyroid, Parathyroid and Adrenal Tissue

Tissue	Histopathology	Positivity/No. of Cases
Thyroid	Normal	0/8
	Hashimoto's	0/3
	Graves Disease	0/1
	Nodular Colloid Goiter	0/6
	Follicular Adenoma	2/4
	Mixed Papillary and Follicular Carcinoma	6/8
	Medullary Carcinoma	1/1
	Metastatic Squamous Carcinoma	1/1
Parathyroid	Normal	0/3
	Hyperplasia	0/2
	Parathyroid Adenoma	2/5
Adrenal	Normal	0/5
	Pleochromocytoma	2/2

Two out of four follicular adenomas showed weak cytoplasmic staining (Figure 8).

One follicular carcinoma was moderately positive, and one papillary carcinoma was weakly positive. CEA in both instances was localized in the perinuclear region. Mixed papillary and follicular carcinoma showed weak to moderate cytoplasmic staining (Figure 16).

2. Parathyroid Gland

Normal and hyperplastic parathyroid glands were negative. Two parathyroid adenomas (oxyphil cells) were weakly positive, but the three clear cell adenomas were negative (chief cells).

3. Adrenal Gland

There was no staining of normal adrenal gland cortex or medulla. Two pheochromocytoma showed moderate cytoplasmic staining with negative nuclear staining (Figure 17). Adjacent normal adrenal tissue was negative or very weakly positive (Figure 17).

C. Urinary System

The result of immunohistochemical staining for CEA is shown in Table III.

1. Kidney

Glomeruli from normal kidneys and from glomerulonephritis did not stain. There was weak cytoplasmic staining in the proximal and distal convoluted tubules, and this reaction increased in one case where the tissue was close to a tumor (renal cell carcinoma).

Three out of five renal cell carcinomas were weakly to moderately positive (Figure 18) and staining intensity had no relation to

TABLE III

Immunohistochemical Staining of CEA
in Normal and Neoplastic Urinary System Tissue

Tissue	Histopathology	Positivity/No. of Cases
Renal Glomeruli	Normal	0/6
Renal Tubules	Normal	5/6
	Pyonephrosis	0/1
	Renal Adenocarcinoma	3/5
Dysplastic Kidney	Multicystic Dysplasia	0/1
	Mesangial Proliferative Glomerulonephritis	0/3
	Foot Process Disease	0/1
Ureter and Urinary Bladder	Normal Transitional Epithelium	10/15
	Transitional Cell Carcinoma	8/9
	Mucinous Adenocarcinoma	1/2

tumor differentiation or presence or absence of metastases to adjacent lymph nodes.

2. Ureter and Urinary Bladder

Ten out of fifteen normal transitional epithelium showed weak positive membrane staining and negative to weak positive cytoplasmic staining.

One of two invasive mucinous adenocarcinoma showed moderate cytoplasmic staining and negative mucus staining. Eight out of nine transitional cell carcinoma (I-V) showed moderate cytoplasmic staining and membrane staining (Figure 19).

D. Lung

The results of immunohistochemical staining for CEA is shown in Table IV.

Normal lung parenchyma, hyaline membrane disease of infancy, cystic adenomatoid malformation and necrotic granuloma (presumably infections) were all negative.

One carcinoid tumor showed very weak cytoplasmic positivity.

One squamous carcinoma showed negative staining. One metastatic teratocarcinoma was negative except a few epithelial cells (probably immature bowel) showed weak fine cytoplasmic staining.

A primary pulmonary adenocarcinoma showed moderate to strong perinuclear cytoplasmic and glycocalyx positivity, also the mucinous adenocarcinoma showed strong cytoplasmic positivity with mucous produced by the tumor cells did not stain.

TABLE IV

Immunohistochemical Staining of CEA
in Normal and Neoplastic Lung Tissue

Tissue	Histopathology	Positivity/No. of Cases
Lung	Normal	0/7
	Hyaline Membrane Disease	0/4
	Cystic Adenomatoid Malformation	0/1
	Necrotic Granuloma	0/2
	Rheumatoid Lung	0/1
	Carcinoid Tumor	1/1
	Poorly Differentiated Small Cell Carcinoma	3/4
	Squamous Carcinoma	0/1
	Teratocarcinoma (Metastatic)	0/1

TABLE V

Immunohistochemical Staining of CEA
in Normal and Neoplastic Breast Tissue

Tissue	Histopathology	Positivity/No. of Cases
Breast	Normal	0/8
	Duct Papilloma	2/2
	Mammary Dysplasia	2/7
	Duct Carcinoma	6/6
	Adenocarcinoma	3/3

Three out of four anaplastic small cell carcinoma were strongly positive (Figure 20). Normal parenchyma adjacent to the tumor was negative.

E. Breast

The result of the immunohistochemical staining is shown in Table V.

Normal breast tissue was completely negative.

Duct papilloma, duct carcinoma (Figure 21), Paget's disease and adenocarcinoma showed from weak to strong cytoplasmic positivity and membrane staining. Duct papilloma staining in general was weak positive. The intensity of staining varied from weak in duct papilloma to strong in infiltrating duct carcinoma and Paget's disease.

Two out of seven dysplastic breast tissues showed weak cytoplasmic positivity and weak membrane staining of the duct cells.

Normal tissue adjacent to tumor was negative.

F. Female Reproductive System

The results of immunohistochemical staining is shown in Table VI.

1. Ovary

Two benign cystic teratomas showed very weak positivity, in the glandular epithelium, the staining is fine, cytoplasmic granules.

Malignant endodermal sinus tumor showed weak positive cytoplasmic staining.

Active corpus luteum showed very strong cytoplasmic CEA staining with negative nuclei.

TABLE VI

Immunohistochemical Staining of CEA
in Normal and Neoplastic Female Reproductive System

Tissue	Histopathology	Positivity/No. of Cases
Ovary	Corpus Luteum	1/1
	Benign Cystic Teratoma	2/2
	Benign Mucinous Cystadenoma	0/1
	Malignant Endodermal Sinus Tumor	1/1
	Papillary Serous Cystadenocarcinoma	1/1
	Mucinous Cystadenocarcinoma	1/1
Tubes	Normal	0/3
Uterus	Normal Endometrium	5/5
	Normal Endocervix	2/3
	Normal Endocervix Gland	0/7
	Adenomyomatous Polyp	1/1
	Fibroepithelial Polyp of Endocervix	0/1
	CIN (III)	4/4

A benign mucinous cystadenoma was negative while a mucinous cystadenocarcinoma showed moderate cytoplasmic and membrane staining and no mucin staining. A papillary serous cystadenocarcinoma was weakly positive with fine cytoplasmic staining in epithelium.

2. Tubes

No staining in all three cases.

3. Uterus

The normal endometrium showed weak cytoplasmic epithelial and membrane staining.

Two out of three exocervical squamous epithelium showed moderate membrane and weak cytoplasmic staining. The basal layer cells were negative.

Severe dysplastic epithelium (CIN III) showed strong membrane and weak cytoplasmic staining within tumor cells. Basal layer cells were negative and the intensity of staining increased toward superficial cells (Figure 22).

With carcinoma in situ showed strong cytoplasmic staining and moderate membrane staining. The basal layer cells were negative to weak positive, also staining increased with maturity (Figure 23).

Endocervical glands and a benign endocervical polyp were completely negative (Figure 23).

An adenomyomatous polyp showed weak cytoplasmic positivity in the glandular epithelium.

G. Male Reproductive System

The result of immunohistochemical staining is shown in Table VII.

TABLE VII

Immunohistochemical Staining of CEA
in Normal and Neoplastic male Reproductive System

Tissue	Histopathology	Positivity/No. of Cases
Prostate	Normal	6/10
	Hyperplasia	3/5
	Adenocarcinoma	12/12
Testis	Normal Seminiferous Tubules	0/2
	Leydig Cells	2/2
	Teratocarcinoma	0/1
	Metastatic Seminoma	1/1
Seminal Vesicle	Normal	0/1

1. Prostate

Six out of ten normal prostates showed weak cytoplasmic and membrane staining. Prostatic adenocarcinoma in the prostate (Gleason Grade 1-5) showed moderate cytoplasmic and membrane staining, the positivity did not change drastically by the grade of adenocarcinoma. Normal prostate adjacent to the tumor was negative or weakly positive. Three out of five hyperplastic prostates showed weak cytoplasmic staining.

2. Seminal Vesicle

One normal seminal vesicle showed negative staining.

3. Testis

Testicular tubules were negative (seminiferous tubules). Interstitial Leydig cells showed strong cytoplasmic staining (Figure 24).

One metastatic seminoma showed moderate cytoplasmic staining and weak membrane staining. One teratocarcinoma of the testis was negative (Figure 25).

In all types of tissue tested there was minimal connective tissue staining and negative staining in the nucleus, nerve and lymphoid tissue.

IV. DISCUSSION AND CONCLUSION

A. Gastrointestinal Tract and Associated Glands

CEA was demonstrated in the epithelial cells of many gastrointestinal organs, including normal stomach, small intestine, large bowel, liver and salivary glands, and in neoplasms, both benign and malignant, arising from these organs, as well as in polyps found in stomach and colon (Wiley et al., 1981, Isaacson et al., 1976, Klein et al., 1981, Rognum et al., 1980, and Wagner et al., 1978). CEA was also demonstrated in inflammatory bowel conditions such as Crohn's disease and ulcerative colitis. These inflammatory diseases showed moderate, fine, epithelial cytoplasmic and membrane glycocalyx staining (Figure 3). CEA staining increased with increased tissue damage. This increase in CEA detection may be due to immature epithelial cell proliferation or epithelial cell damage with the release or exposure of cytoplasmic CEA.

All adenocarcinomas of the colon, including the rectum and stomach, studied demonstrated moderate to strong cytoplasmic and membrane staining. The intensity of staining may be due to cell dedifferentiation with heightened expression of CEA in the dedifferentiated cells, mimicking the expression of CEA in fetal intestinal epithelium.

CEA was not uniform in all tumor cells. A few cells had weak staining, others were moderately or strongly stained. This variability in staining may be due to variation in individual cell metabolic rate or synthesis of CEA. Increased cytoplasmic synthesis of CEA by

tumor cells may be reflected by increased plasma cell levels. Similar increases in plasma CEA may be associated with inflammatory bowel diseases (Goslin et al., 1981, Minton et al., 1978, Mavligit et al., 1978, Mach et al., 1978).

The localization of CEA to the cell surface (probably glycocalyx) (also shown by Fuks et al., 1974, and by Ferritin labelling) and within the cytoplasm of neoplastic tissue suggests that CEA is a secretion product rather than a constituent of the tumor cell membrane. The presence of CEA in and on neoplastic cells may be due to increased synthesis or increased accumulation of CEA by the neoplastic cells or to both.

PAP CEA detection in colorectal adenocarcinomas was not affected by tumor size, although plasma CEA level would likely be higher in patients with larger tumors, since the increased bulk of tumor cells could synthesize and release larger quantities of CEA to the circulation. CEA staining intensity did not change greatly with tumor differentiation and this finding contradicts that of Denk et al., 1972, who postulated that poorly differentiated carcinomas would have negative CEA and high CEA levels would be present only in well differentiated carcinomas. The intensity of CEA staining in colorectal adenocarcinomas did not vary with depth of invasion (as measured by Dukes, A and B criteria) or with the presence or absence of lymph node metastases (Duke's C). Adenocarcinoma metastatic to lymph nodes had staining intensity similar to the primary tumor.

Moderately well differentiated carcinoma of the esophagus demonstrated weak cytoplasmic and membrane staining, a property not

shared by normal epithelium. One squamous carcinoma of the rectum demonstrated weak cytoplasmic and membrane staining.

Non-neoplastic tissue adjacent to the tumor showed weak to moderate cytoplasmic staining, particularly in superficial epithelium while the deep glandular tissue showed negative to very weak positive membrane staining. This membrane staining (glycocalyx) could be a product of CEA secretion by the tumor with absorption of CEA by normal glycocalyx to increased synthesis by epithelial cells influenced by their proximity to the tumor. Weak membrane and cytoplasmic CEA staining detected in normal gastrointestinal epithelium in small amounts, even in adults, since CEA production is not lost completely with maturity (Wagner et al., 1978, Primus et al., 1981). Stimulation of normal epithelium of this sort by contiguous tumor may increase CEA synthesis or expression.

Adenomas (tubular, tubulovillous, villous) and juvenile polyps all demonstrated weak to moderate cytoplasmic and membrane staining. It has been hypothesized that an increase in staining intensity in large bowel adenomas may predict future malignancy (Isaacson et al., 1976, Primus et al., 1981, Wagner et al., 1978). The results obtained in this study, in which staining increased from slight in normal large bowel epithelium through weak to moderate in large bowel adenomas to moderate to heavy in carcinomas, tend to support that hypothesis but the present data is insufficient to draw firm conclusions.

Two of three pleomorphic adenomas of salivary gland origin showed weak cytoplasmic and membrane staining and the third was negative. One anaplastic carcinoma showed moderate cytoplasmic and

membrane staining and one poorly differentiated squamous carcinoma (primary in salivary gland) showed weak cytoplasmic and membrane staining. Normal tissue adjacent to malignant tumor showed weak perinuclear and membrane staining of the intercalated ducts and apical staining within some of the acini. Salivary gland malignancies are probably derived from undifferentiated duct cells and these cell types are presumably related to intercalated ducts. CEA in normal intercalated duct cells may represent a product of duct synthesis of CEA, analogous to the proposed stimulation of normal large bowel epithelium by contiguous tumor, to absorption of CEA secreted by the malignant tumor by intercalated ducts, glycocalyx, or to excretion by normal duct cells of CEA produced or accumulated by the malignant tumor. CEA in intercalated duct cells of normal salivary gland may explain the presence of CEA in the saliva of some normal people and in patients with salivary gland malignancies (Caselitz et al., 1981, McDicken and Scott, 1981, Caselitz et al., 1981).

Normal pancreas exocrine was negative except for weak cytoplasmic and membrane staining of intralobular ducts, and weak acinar cytoplasmic staining in chronic pancreatitis. The localization of CEA in pancreatic ducts may explain the presence of CEA in pancreatic juice (Carr-Locke, 1980, Kalvins, 1981).

Islands of Langerhans showed moderate cytoplasmic staining. This could represent a cross-reaction with a substance which is antigenically similar or identical to CEA or may be CEA synthesized or accumulated by the islets. One metastatic anaplastic small cell carcinoma, most likely from the lung, was negative.

Normal hepatocytes always showed weak cytoplasmic perinuclear and very weak membrane staining. Hepatocyte CEA may be an indication of liver involvement in CEA clearance from the plasma (Gerber et al., 1978). CEA staining intensity was increased with mild cholestasis, alcoholic liver cirrhosis, and in tumor cell of liver cell adenoma and hepatoma. This may be due to inability of the damaged or neoplastic cell to excrete CEA or to increased CEA production by the damaged or neoplastic cell. Staining intensity of normal hepatic parenchyma did not vary with the presence or absence of a neoplasm.

Normal gallbladder was negative. One adenosquamous carcinoma (primary in gallbladder) showed weak cytoplasmic and membrane staining which increased with increase keratinization of the tumor. This may be due to a nonspecific cross-reaction with keratin or keratin precursors.

Epithelial mucin was present in most tissue blocks examined and in all of these mucin was negative for CEA staining. These negative results tend to prove that there is no cross-reaction between CEA and NCA II, the latter a prominent component of epithelial mucin.

B. Thyroid, Parathyroid and Adrenal Glands

CEA was not detected in normal thyroid, parathyroid or adrenal glands. Non-neoplastic conditions of the thyroid such as Hashimotos thyroiditis, Grave's disease and multinodular colloid goiter were negative. Two of four follicular adenomas showed weak cytoplasmic and membrane staining.

One pure follicular carcinoma and six of eight mixed papillary and follicular carcinomas showed weak to moderate cytoplasmic and membrane staining in agreement with Calmettes et al., 1982. The staining did not vary with the degree of differentiation or the size of the tumor. Staining was variable from one cell to another, probably depending on the metabolic activity of the individual cell. One papillary carcinoma showed only weak positive cytoplasmic and membrane staining. One medullary carcinoma showed moderate cytoplasmic and membrane staining. One metastatic squamous carcinoma showed weak cytoplasmic and moderate membrane staining and staining intensity increased with the degree of keratinization.

Parathyroid hyperplasia was negative. Two parathyroid adenomata (oxyphil cells) showed weak cytoplasmic staining and three clear cell adenomas were negative. The significance of staining variability in parathyroid adenomas is not clear. It is possible the clear cell adenomas were actually "clear cell hyperplasia."

Normal tissue adjacent to neoplastic tissue in the thyroid and parathyroid was negative.

Two pheochromocytomas of adrenal gland showed moderate cytoplasmic staining. This may be due to increased production or inability to excrete CEA with increased cytoplasmic accumulation. Normal adrenal tissue adjacent to neoplastic tissue was negative or very weak cytoplasmic staining.

C. Urinary System

Renal glomeruli in normal and non-neoplastic abnormal kidney such as multicystic dysplasia, mesengial proliferative glomerulonephritis, and foot process disease were negative.

Renal tubules in five of six kidneys showed weak cytoplasmic and membrane staining and this reaction intensified when the normal tissue was adjacent to neoplastic tissue. This may indicate an active role for renal tubules in the excretion of CEA. A single pyonephrosis was negative glomerular and tubular staining.

Three of five renal cell carcinomas showed moderate cytoplasmic and membrane staining. The staining reaction did not correlate with tumor differentiation, the size of the renal tumor or the presence of metastases. Ten normal bladder transitional epithelium (urothelium) of fifteen showed weak to moderate cytoplasmic and membrane staining. The membrane staining was more prominent than the cytoplasmic (Wahren, 1978, Shevchuck et al., 1981). Eight of nine transitional cell carcinomas of various grades (I-IV) showed moderate cytoplasmic and membrane staining (Shevchuck et al., 1981, Wahren et al., 1977, Wahren, 1978). The cytoplasmic staining intensity in transitional cell carcinoma was more marked than in adjacent normal transitional epithelium. The increase in cytoplasmic staining may be due to increased synthesis of CEA by the neoplastic cells, or inability of the neoplastic cells to excrete or clear CEA from their cytoplasm. Staining intensity did not vary greatly between different grades of transitional cell carcinoma, in disagreement with Wahren, 1978. One of two mucinous adenocarcinomas showed moderate cytoplasmic and membrane staining. The mucin did not stain.

D. Lung

Normal lung parenchyma, parenchyma adjacent to neoplasms, hyaline membrane disease of infancy, cystic adenomatoid malformation and necrotic granuloma (presumably infections) were all negative. One carcinoid tumor showed weak cytoplasmic CEA staining. Three of four small cell anaplastic carcinomas showed strong positive staining and this result is in agreement with Shested et al., 1981, O'Brien et al., 1980. Poorly differentiated squamous carcinoma in agreement with Pascal et al., 1977 was negative. One metastatic teratoma carcinoma was also negative and this may be due to absence of gastrointestinal epithelium in the tumor.

E. Breast

Normal breast tissue was negative. Two of seven cystic diseases of the breast showed weak cytoplasmic and membrane staining of the intralobular ducts and apical staining in a few acini. CEA is apparently produced or retained in at least some breast tissue and this may help explain CEA detection in the milk of some women (Kuhana et al., 1981). The presence of CEA in milk, breast secretions or cystic disease may indicate the possibility of future malignancy. This hypothesis requires long-term study.

Duct papilloma showed weak cytoplasmic and membrane staining and some lumenal staining, while duct carcinoma and adenocarcinoma showed stronger reaction in the same regions (Kahana et al., 1981, Wells and Hasleton et al., Walker, 1980, Shoushan and Lyssiotis,

1978). Positivity did not vary with metastases, tumor size or tumor differentiation.

Normal adjacent breast tissue was negative.

F. Female Reproductive System

One active corpus luteum showed strong cytoplasmic staining, which could be a false positive reaction due to cross-reaction of anti-CEA with some hormones, or it could be that CEA is normally expressed by the corpus luteum. Two benign cystic teratomas showed weak cytoplasmic and membrane staining in the epithelium (endoderm). Benign mucinous cyst adenoma was negative while a single malignant mucinous cyst adenocarcinoma showed moderate cytoplasmic and membrane staining. Papillary serous cystadenocarcinoma and malignant endodermal sinus tumor showed weak cytoplasmic and membrane staining (Primus et al., 1981, Boer and Nayman, 1981).

Normal fallopian tube epithelium was negative.

Normal endometrium showed weak cytoplasmic and membrane CEA staining. Normal exocervix showed weak cytoplasmic and moderate membrane staining. Cytoplasmic staining was more intense in CIN III. The basal epithelial layer was consistently negative. Normal endocervical epithelium was negative even when adjacent to CIN III (Cohn et al., 1982).

An adenomyomatous polyp showed weak cytoplasmic and membrane staining indicating derivation from endometrium while a fibroepithelial polyp of endocervix was negative. Staining of CIN increased with maturation or keratinization (Nagell et al., 1982). The presence of CEA in the better differentiated areas of squamous cell carcinoma of

the CIN seems disturbing initially since most ectodermal tumors should not be expected to produce CEA. Yet, if one regards these tumors as endodermally derived malignant neoplasms, that exhibits metaplasia and that have not totally lost the morphological expression of cellular differentiation, then the data are in keeping with the hypothesis that states endodermally derived tumors will produce CEA (Gold and Freeman, 1965). An alternative explanation for finding CEA or CEA-like material in the keratinizing squamous areas is that CEA cross-reacts with keratin or keratin precursors.

G. Male Reproductive System

Six of ten normal and hyperplastic prostatic tissue biopsies showed weak cytoplasmic and membrane staining. Adenocarcinomas of the prostate showed moderate cytoplasmic and membrane staining. Intensity of stain did not correlate with the grade or the tumor metastases. Normal seminiferous tubules were negative. Leydig cells showed strong cytoplasmic staining. One poorly differentiated teratocarcinoma showed negative results due to lack of endodermal epithelium. One metastatic seminoma showed moderate cytoplasmic and membrane staining (Primus et al., 1982).

The PAP technique is the most sensitive immunoperoxidase method for the immunohistochemical localization of CEA by light microscopy in conventionally processed tissue sections. Cytoplasmic staining noticed in normal and neoplastic tissue is not due to artifact, caused by the diffusion of DAB from the site of oxidation, or by oxidation, or by delayed or incomplete fixation (Isaacson and Levann, 1976).

The result and discussion show that CEA not only is found in endoderm but also in other germ layers. It is not specific to malignant neoplasms but also found in benign neoplasms and in normal tissue of diffeent organs, although to a lesser degree. CEA is also expressed in many inflammatory conditions.

DIRECT PEROXIDASE PROCEDURE

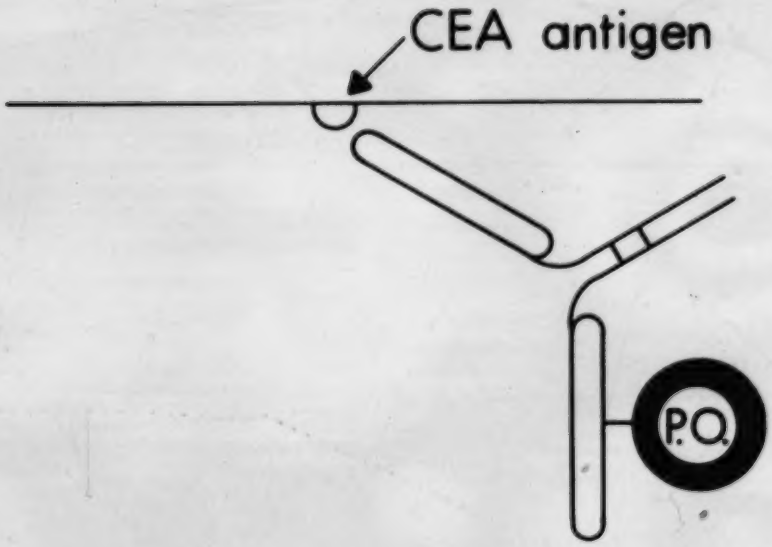

Fig 1.- Direct immunoperoxidase method.
(Modified from Sternberger, 1979).

INDIRECT PEROXIDASE PROCEDURE

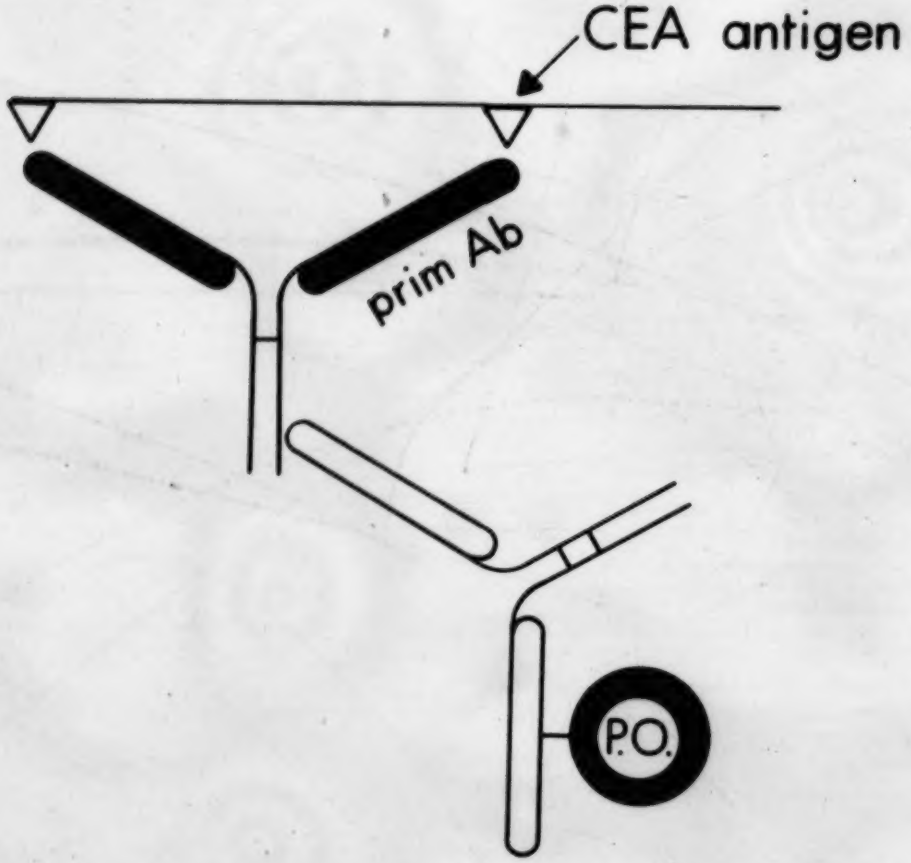

Fig 2.- Indirect immunoperoxidase method.
(Modified from Sternberger, 1979).

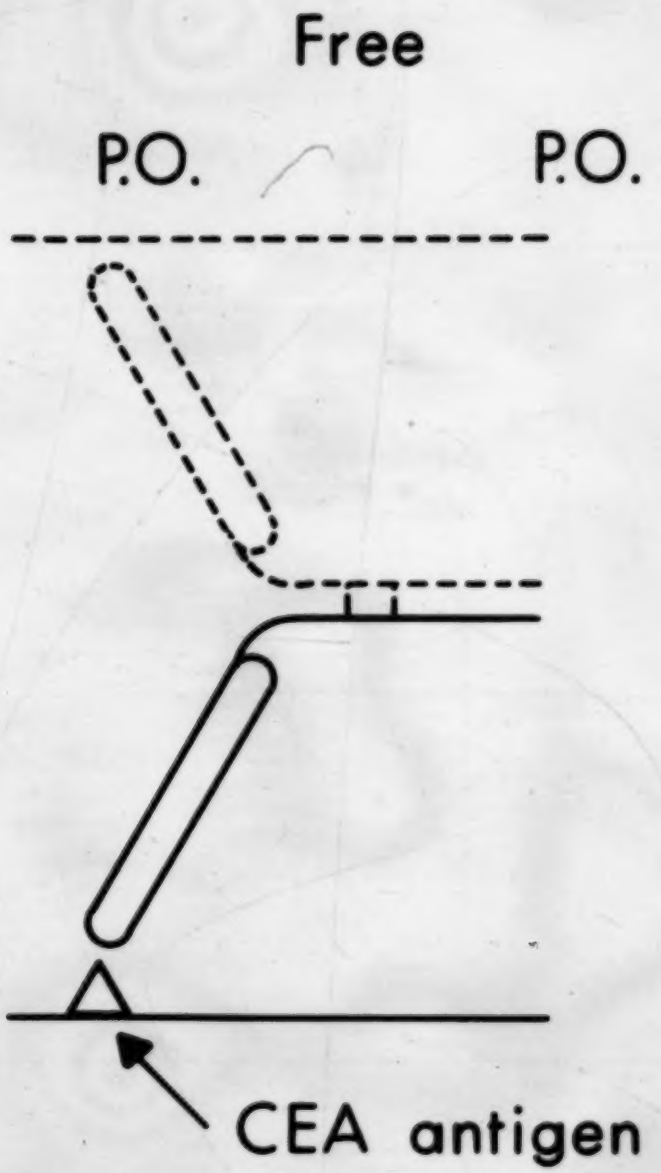

Fig 3. - Hybrid antibody method.
(Modified from Taylor, 1978).

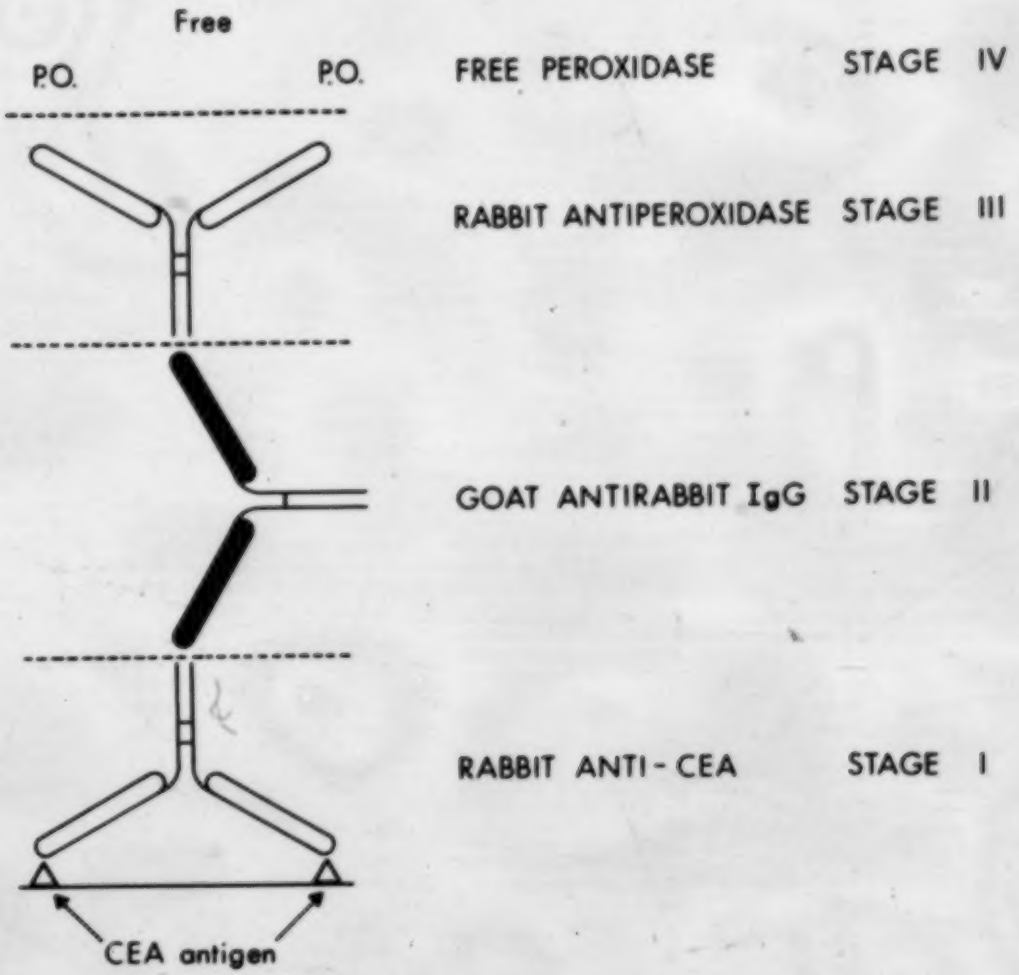

Fig 4.- Enzyme bridge method. (Modified from Taylor, 1978).

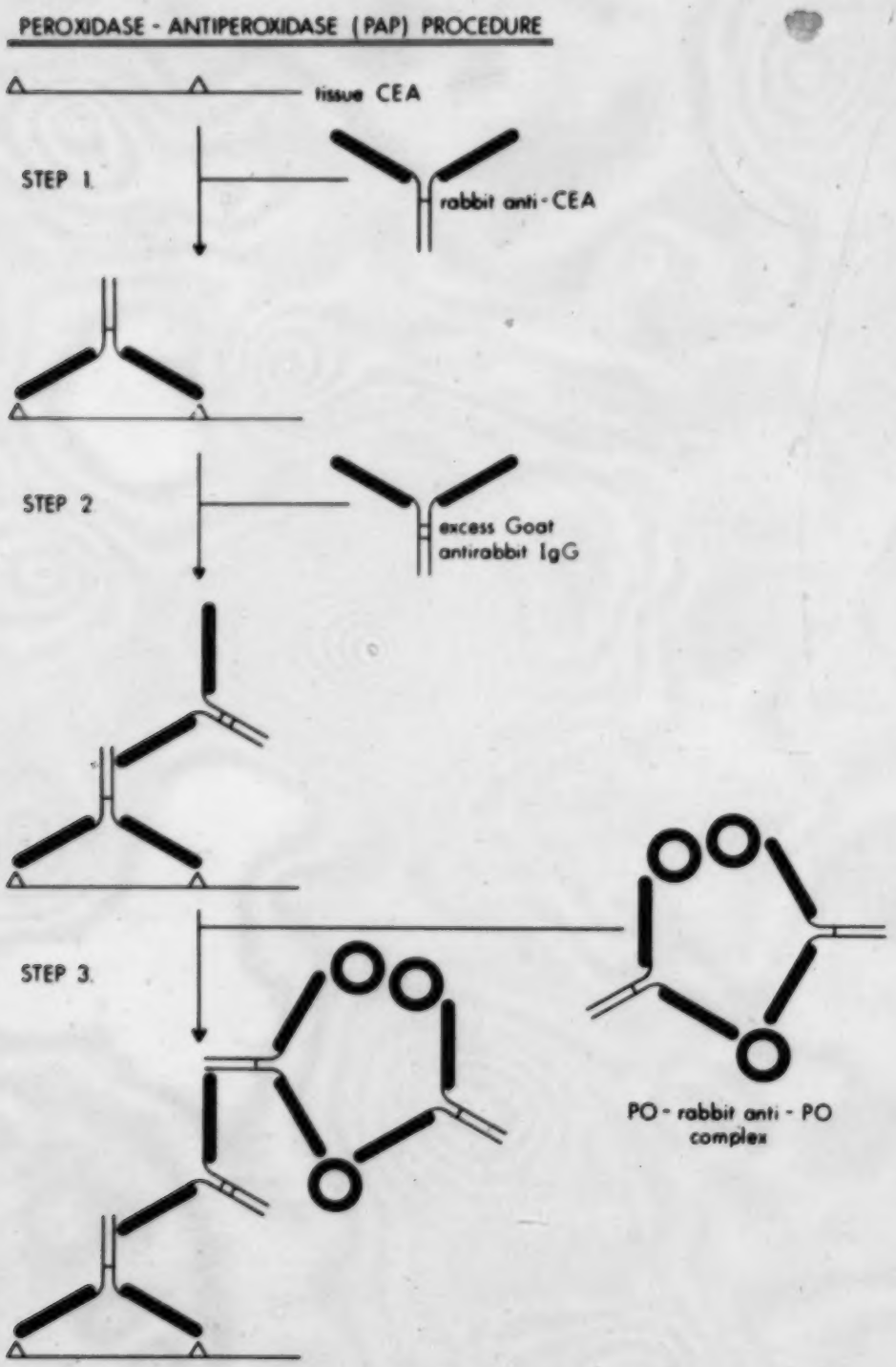

Fig 5 - Peroxidase - antiperoxidase technique. (Modified from Sternberger, 1979)

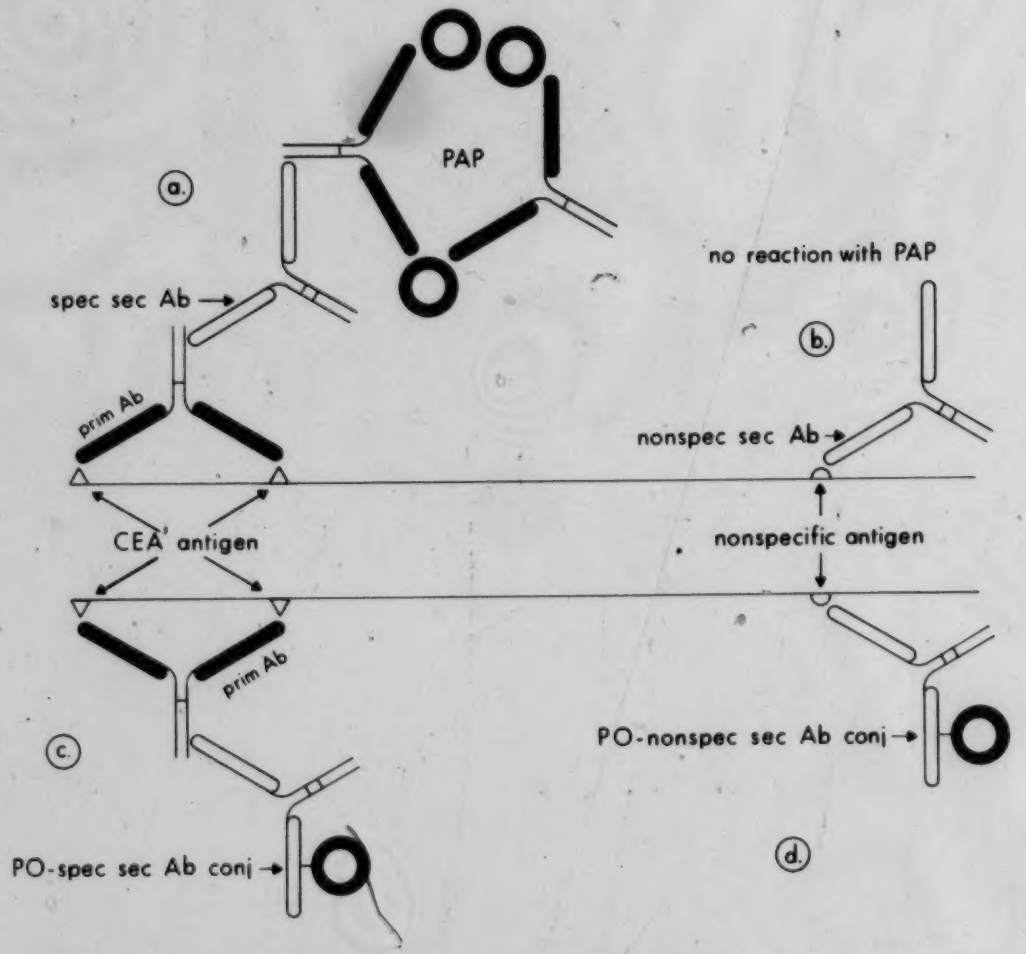

Fig 6. - Nonspecific background with PAP and peroxidase-labeled antibodies.
(Modified from Sternberger, 1979).

Figure 7 Immunoperoxidase staining of a normal, formalin fixed, paraffin embedded small bowel specimen. X 25.

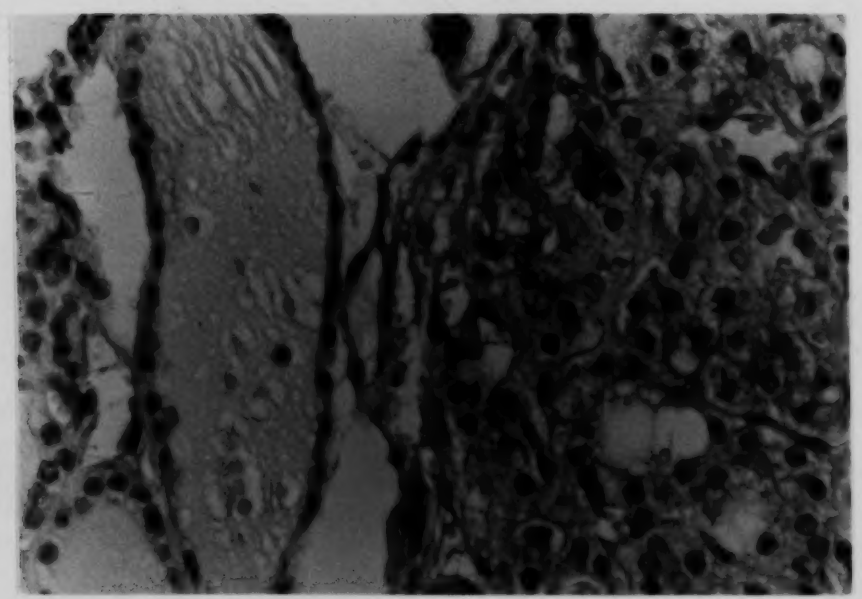

Figure 8 Immunoperoxidase staining of microfollicular adenoma, formalin fixed, paraffin embedded thyroid specimen. X 309. Brownish color indicates CEA localization.

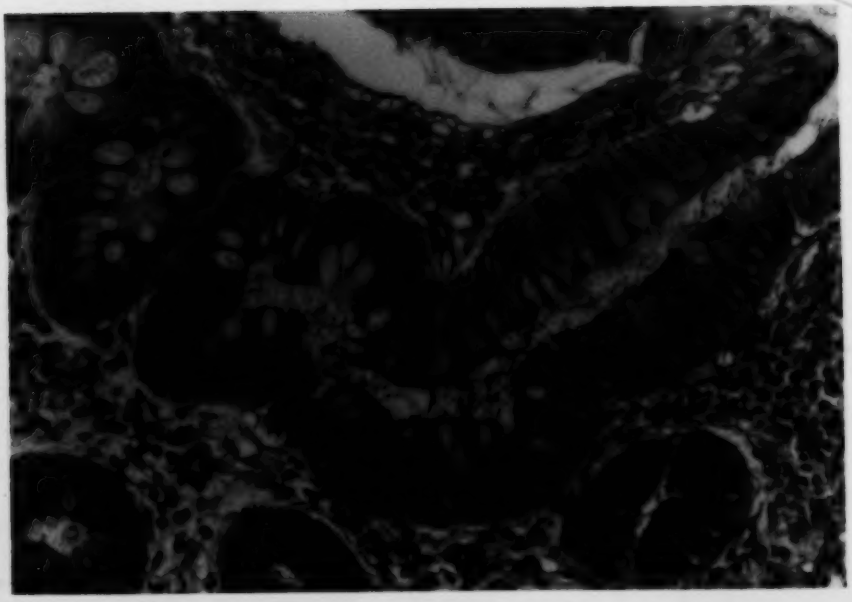

Figure 9 Immunoperoxidase staining of ulcerative colitis, formalin fixed, paraffin embedded colon specimen. X 155. Brownish color indicates CEA localization.

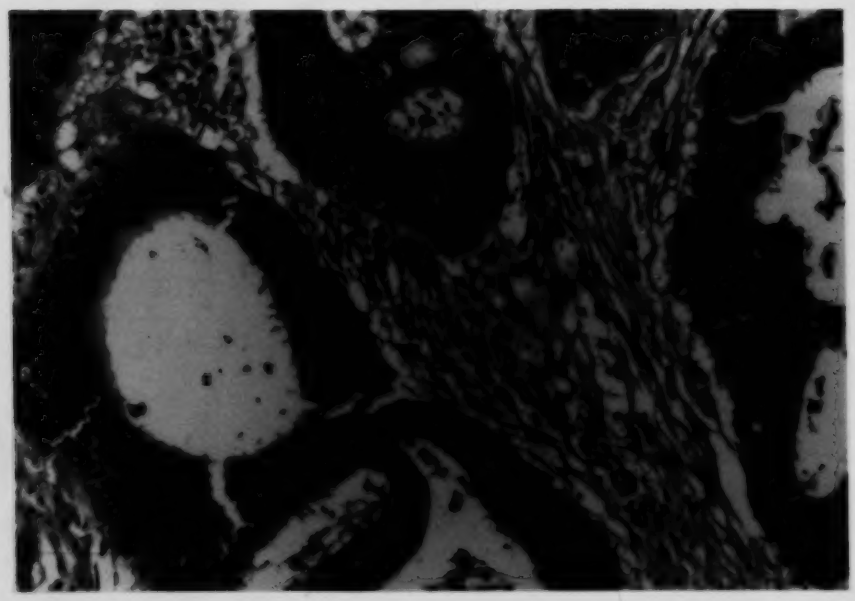

Figure 10 Immunoperoxidase staining of moderately well differentiated adenocarcinoma, Dukes class B, formalin fixed, paraffin embedded colon specimen. X 309. Brownish color indicates CEA localization.

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Figure 11 Immunoperoxidase staining of a tubular adenoma, formalin fixed, paraffin embedded colonic polyp specimen. X 97. Brownish color indicates CEA localization.

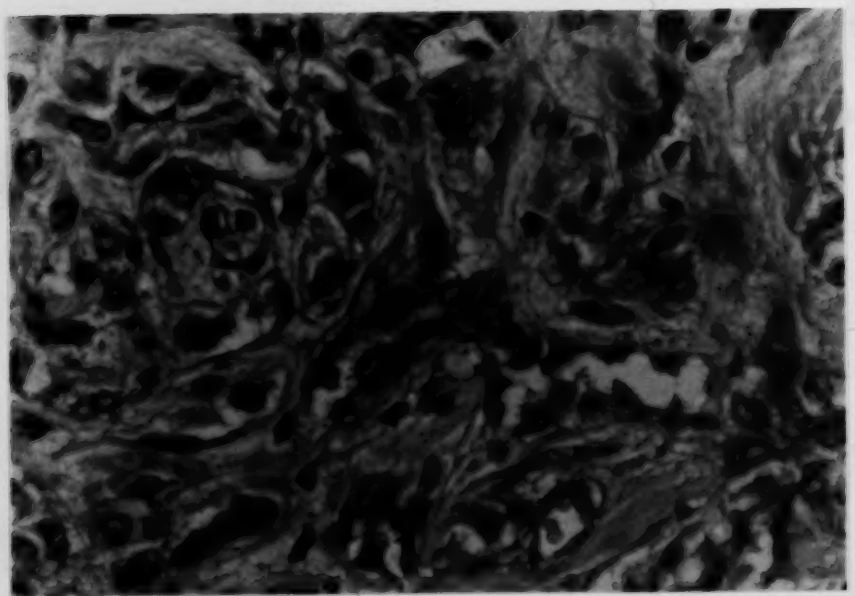

Figure 12 Immunoperoxidase staining of a benign pleomorphic adenoma, formalin fixed, paraffin embedded salivary gland specimen. X 309. Brownish color indicates CEA localization.

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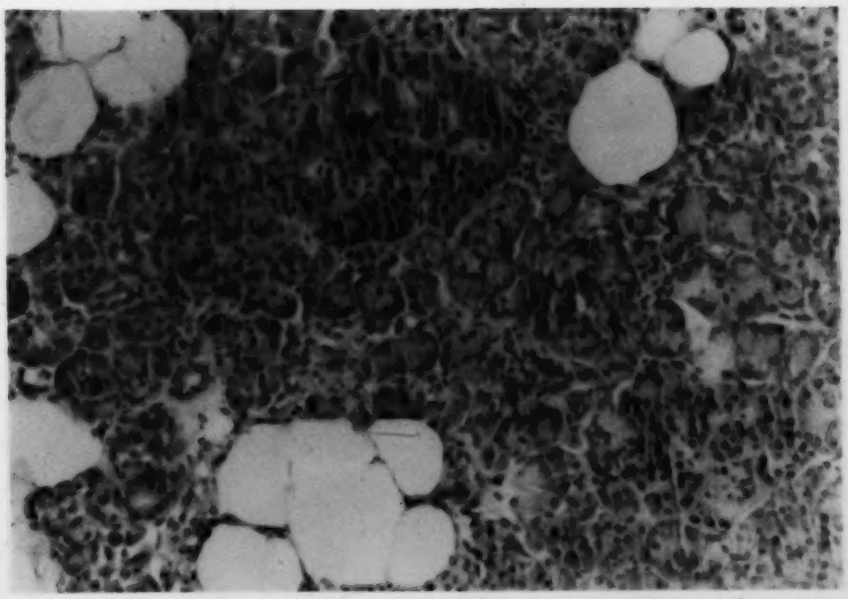

Figure 13 Immunoperoxidase staining of a normal, formalin fixed, paraffin embedded pancreas specimen. X 124. Brownish color indicates CEA localization.

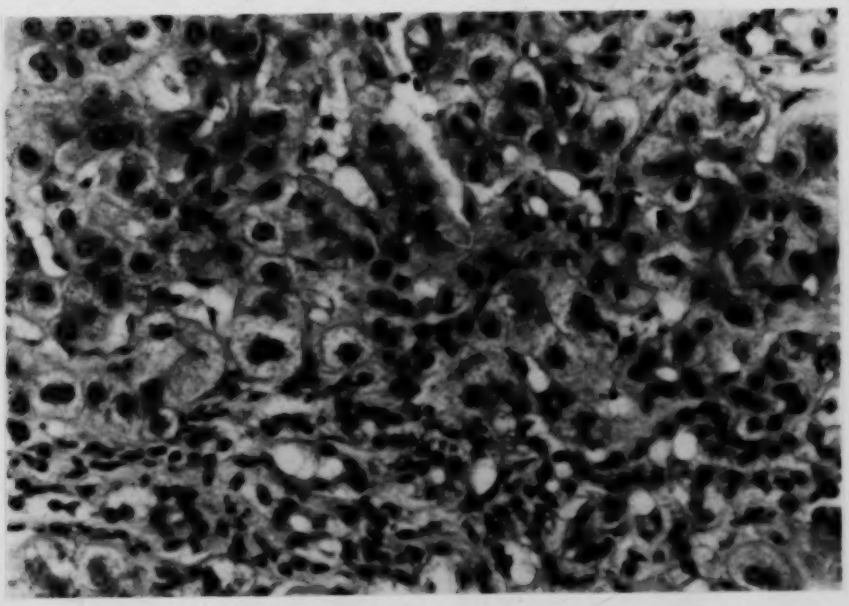

Figure 14 Immunoperoxidase staining of an active cirrhosis, formalin fixed, paraffin embedded liver specimen. X 243. Brownish color indicates CEA localization.

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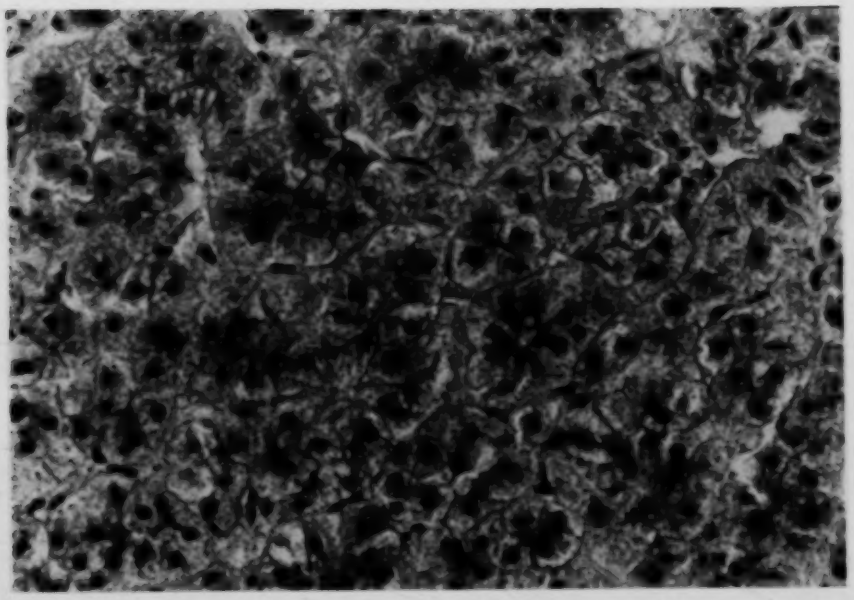

Figure 15 Immunoperoxidase staining of a liver cell adenoma specimen, formalin fixed, paraffin embedded. X 243. Brownish color indicates CEA localization.

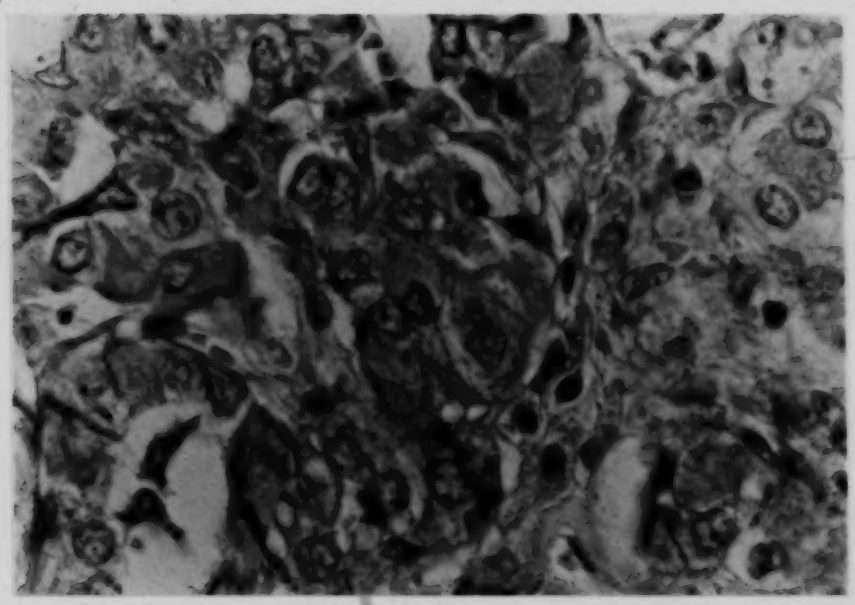

Figure 16 Immunoperoxidase staining of a metastatic mixed papillary and follicular carcinoma, formalin fixed, paraffin embedded thyroid specimen. X 483. Brownish color indicates CEA localization.

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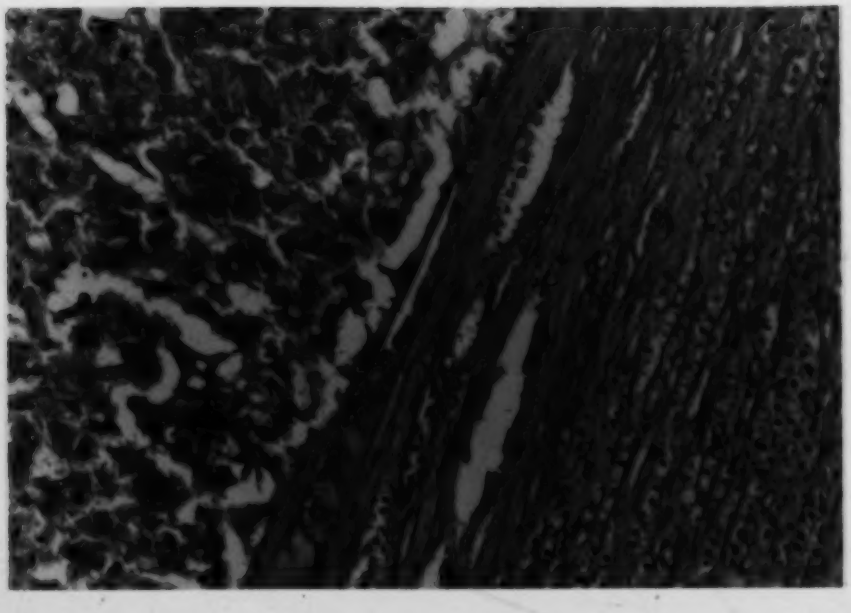

Figure 17 Immunoperoxidase staining of a pheochromocytoma, formalin fixed, paraffin embedded adrenal gland specimen. X 97. Brownish color indicates CEA localization.

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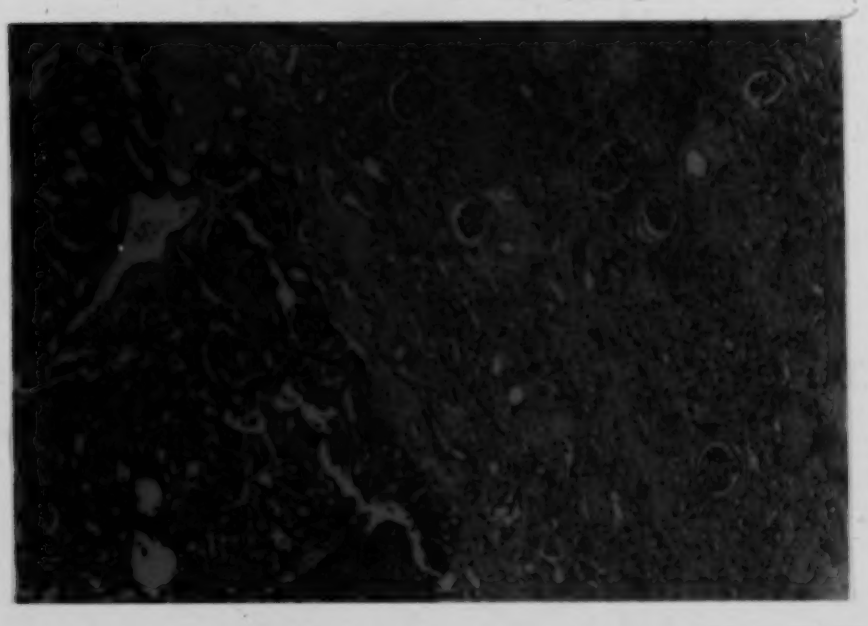

Figure 18 Immunoperoxidase staining of a renal cell carcinoma, formalin fixed, paraffin embedded kidney specimen. X 39. Brownish color indicates CEA localization.

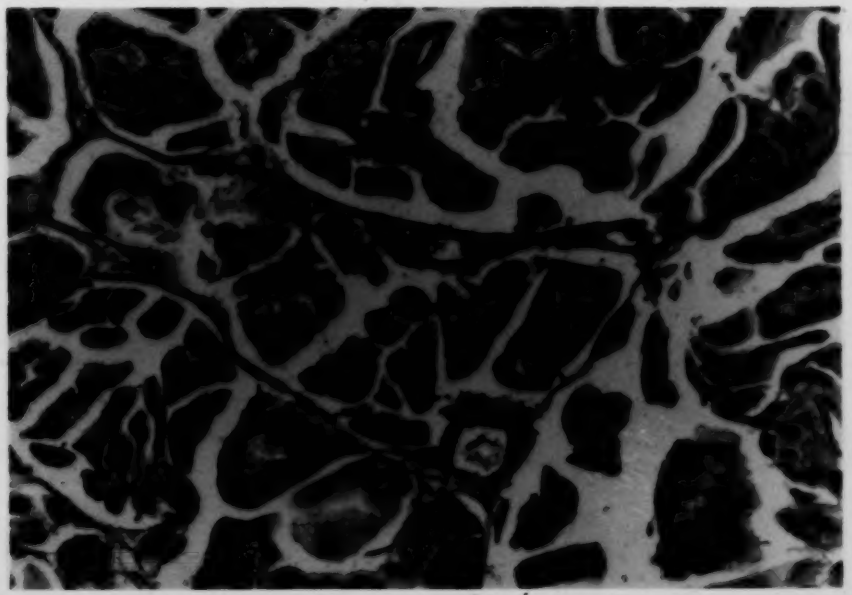

Figure 19 Immunoperoxidase staining of a transitional cell carcinoma, grade IV, formalin fixed, paraffin embedded urinary bladder specimen. X 243. Brownish color indicates CEA localization.

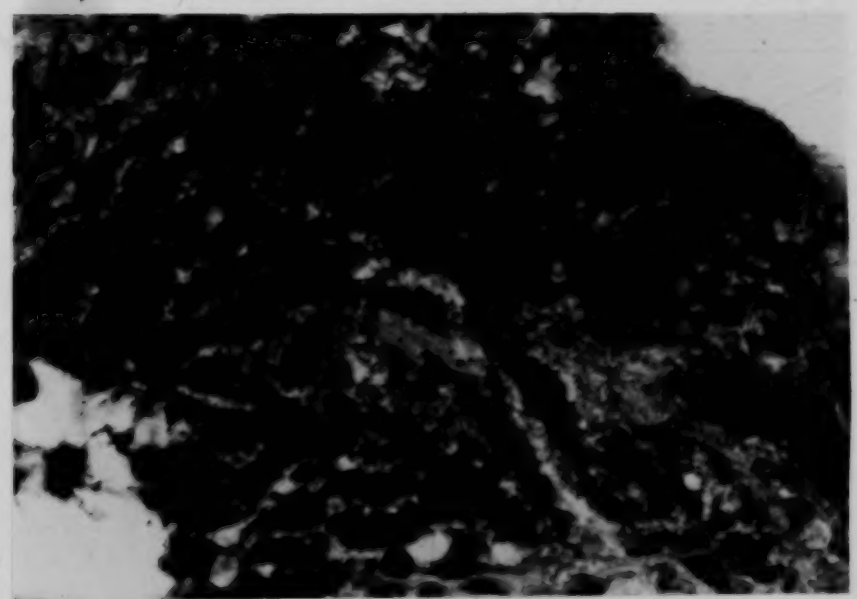

Figure 20 Immunoperoxidase staining of a small cell anaplastic carcinoma, formalin fixed, paraffin embedded lung specimen. X 483. Brownish color indicates CEA localization.

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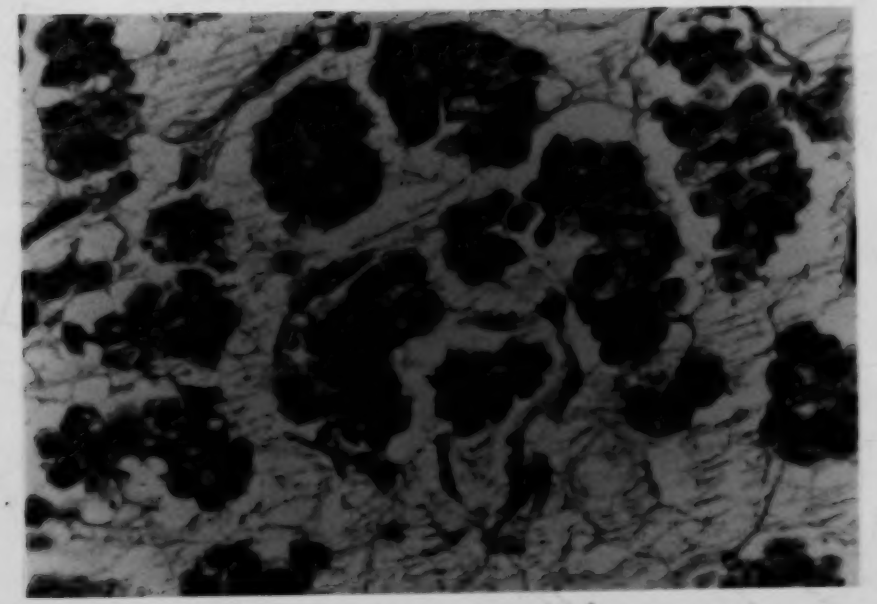

Figure 21 Immunoperoxidase staining of an infiltrating duct carcinoma, formalin fixed, paraffin embedded breast specimen. X 309. Brownish color indicates CEA localization.

Figure 22 Immunoperoxidase staining of a CIN III, formalin fixed, paraffin embedded cervix specimen. X 124. Brownish color indicates CEA localization.

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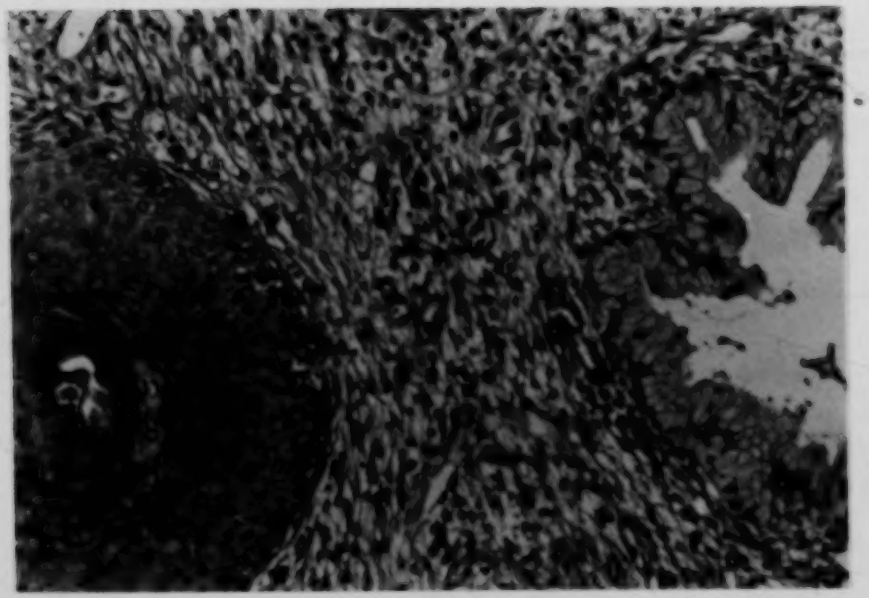

Figure 23 Immunoperoxidase staining of a CIN III, formalin fixed, paraffin embedded cervix specimen. X 124. Brownish color indicates CEA localization.

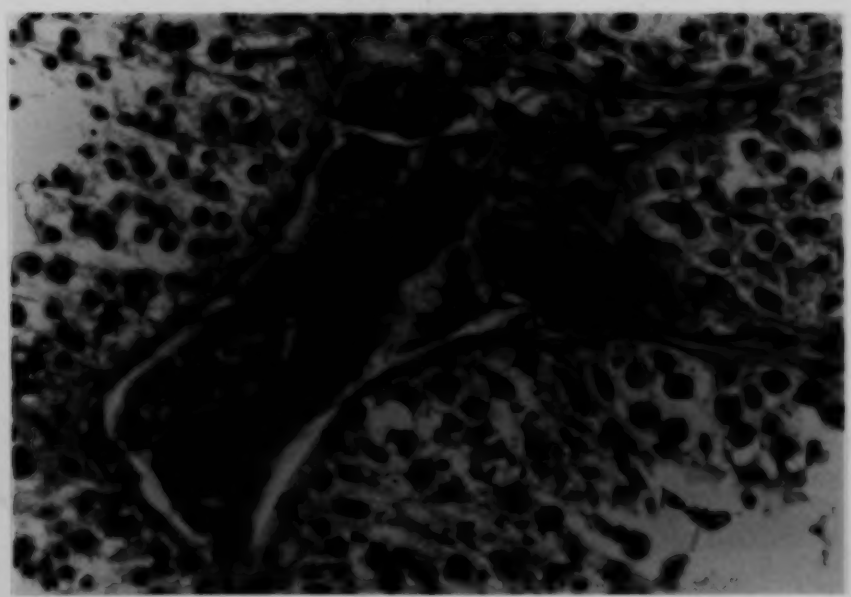

Figure 24 Immunoperoxidase staining of a normal, formalin fixed, paraffin embedded testis specimen. X 309. Brownish color indicates CEA localization.

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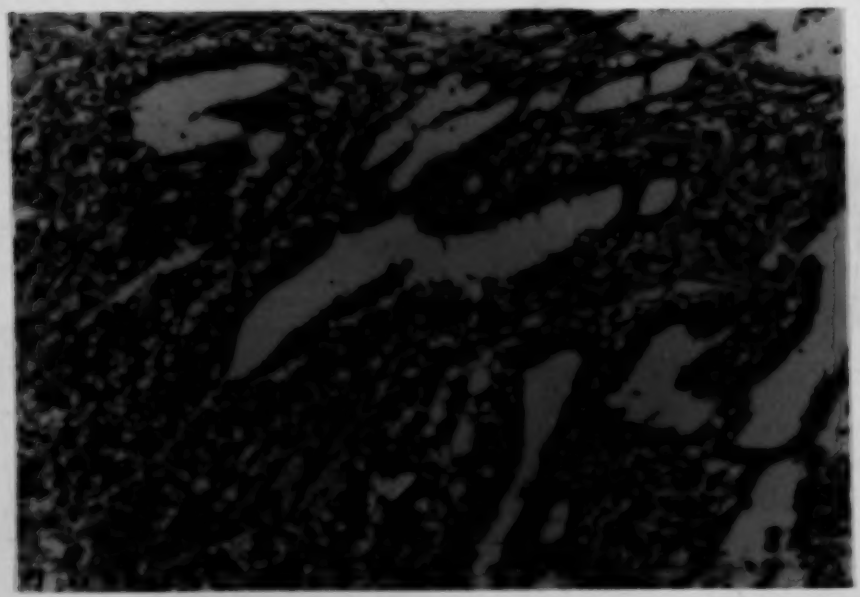

Figure 25 Immunoperoxidase staining of a teratocarcinoma, formalin fixed, paraffin embedded, testis specimen. X 97. Brownish color indicates CEA localization.

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